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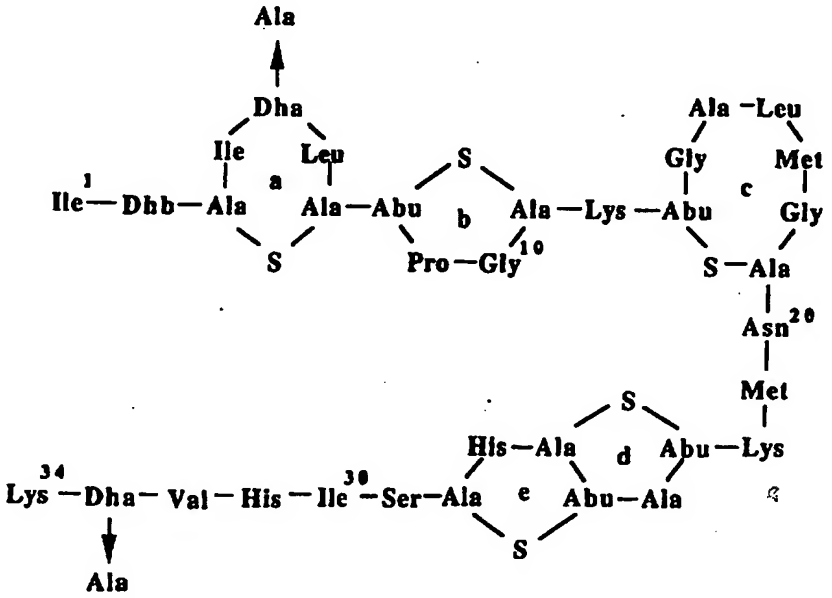

(57) Abstract

A cell which does not contain a natural *nisA* gene but expresses a nisin comprising a variant *nisA* gene wherein the variant *nisA* gene has the same relationship as the natural *nisA* gene to a gene cluster containing the natural *nisA* gene and the genes for nisin modification, secretion and immunity. Preferably the natural, chromosomal *nisA* gene is absent and the cell comprises a variant *nisA* gene at the chromosomal location of the said natural *nisA* gene. Methods of making the cells, and processes for producing nisin, in particular variant nisin, are described.

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Production f variant nisin

The present invention relates to improved methods and bacterial strains for the production of nisin, in particular protein-engineered nisins.

Nisin is a highly modified peptide antibiotic produced, for example, by certain strains of *Lactococcus lactis*. It is of great interest to the food industry because of its efficient antimicrobial activity against a wide range of gram-positive organisms including many spoilage bacteria and food pathogens, for example, *Listeria*, *Clostridia* and *Bacillus* species (see Fowler & Gasson (1990) in *Food Preservatives* (eds N.J. Russell & G.W. Goulds) pages 135-152, Blackie and Sons, Glasgow, UK).

The chemical structure of nisin is well established (Figure 1). It is a member of the family of antibiotics termed lantibiotics. These unusual polycyclic peptides share the structural features of dehydro-residues and intrachain sulphide bridges forming lanthionine and β-methyllanthionine rings. The atypical residues are introduced by post-translational modification of amino acids serine, threonine and cysteine in the primary sequence of a precursor peptide (lantibiotics are the subject of a recent extensive review by Jung (1991) in *Nisins and novel lantibiotics* (eds Jury, G. & Sahl, H.-S.) pages 1-34, ESCOM, Leiden, Netherlands). Biosynthesis of nisin thus involves genes for both the inactive precursor of nisin, known as prenisin, (*nisA*) and also the modifying enzymes responsible for nisin maturation. The mature nisin molecule is based on a sequence of 34 amino acids. The protein encoded by *nis*A includes a 23 amino acid N terminal signal sequence which is cleaved off during secretion of nisin. The conversion of prenisin, encoded by *nis*A, into mature nisin involves cleavage of the leader and the modification of individual amino acids. A *nis*A gene has been cloned and characterised

and shown to have a chromosomal location (see Dodd *et al* (1990) *J. Gen. Microbiol.* **136**, 555-566). A number of additional genes involved in the enzymatic modification of prenisin, translocation and immunity are encoded by nisin producing strains (Kuipers *et al* (1993) *Eur. J. Biochem.* **216**, 281-291; Engelke *et al* (1994) *Appl. Environ. Microbiol.* **60**, 814-825).

Established protein engineering techniques can be used to introduce changes to the amino acid sequence of nisin. This involves modifying the coding region of the nisin structural gene, *nisA*, for example by site-directed or random mutagenesis. Expression of these changes is complicated by the fact that nisin is post-translationally modified.

Variant nisins may be constructed by the expression of variant *nis*A genes in a host strain which encodes the necessary maturation machinery, and thus can process the modified precursor peptide. One approach is to transform a nisin producing strain with a recombinant plasmid encoding a variant *nis*A gene. In this background the host's maturation enzymes are available to process both the resident prenisin and its plasmid-encoded variant. A strategy of this type has been reported for a strain that carries the wild-type nisin transposon (Kuipers *et al* (1991) in *Nisins and novel lantibiotics* (eds Jung, G. & Sahl, H.-S.), pages 250-259, ESCOM, Leiden, Netherlands). However, the disadvantage of this system is that both the host's nisin and the engineered variant are synthesised together, making complex chemical separation procedures necessary prior to analysis of the properties of the novel peptide. Such a procedure would be particularly undesirable for industrial scale production of a variant nisin.

WO 93/20213 describes a process for producing a variant nisin from

Lactococcus in the absence of natural nisin in which a plasmid-borne variant *nisA* gene (which encodes the variant nisin) is introduced into a strain of Lactococcus which does not secrete its natural *nisA* nisin (because the *nisA* gene has been inactivated) but is capable of expressing genes for nisin modification, immunity and translocation out of the cell.

WO 92/18633 discloses plasmid-based systems for the expression of variant nisins from the *nisZ* gene (or mutants thereof) in Lactococcus strains that do not produce natural *nisA* nisin.

Unexpectedly we have found that by replacing the natural, chromosomal copy of the *nisA* gene (or at least a part thereof) with a variant *nisA* gene (or part thereof) we can produce surprisingly high levels of nisin, particularly variant nisins, from Lactococcus. Thus, the present invention provides improved methods and organisms for producing variant nisins with greater efficiency.

One aspect of the invention provides a method for making a cell which does not contain a natural *nisA* gene but expresses a nisin comprising the step of providing a cell with a variant *nisA* gene and genes for nisin modification, secretion and immunity wherein the variant *nisA* gene has the same relationship as the natural *nisA* gene to the gene cluster containing the natural *nisA* gene and the genes for nisin modification, secretion and immunity.

By "providing a cell with a variant *nisA* gene and genes for nisin modification, secretion and immunity" we include inserting a variant *nisA* gene into a cell that already contains genes for nisin modificati n, secretion and immunity as well as inserting into a cell at the same time a variant *nisA* gene plus genes for nisin modification, secretion and

immunity.

The gene cluster containing the genes encoding pre-nisin A (which is processed to form nisin A) and the genes for nisin modification, secretion and immunity from *Lactococcus lactis* (*nisABTCIPRK*) are described in Kuipers *et al* (1993) *Eur. J. Biochem.* **216**, 281-291 and Engelke *et al* (1994) *Appl. Environ. Microbiol.* **60**, 814-825 incorporated herein by reference.

The *nisA* gene is the gene that encodes pre-nisin (pre-nisin includes a 23 amino acid N terminal signal sequence which is cleaved off during secretion); *nisB* and *C* are believed to be involved in reactions which modify the pre-nisin formed directly from expression of the *nisA* gene; *nisT* is similar to a transport ATPase and is involved in translocation of nisin out of the cell; *nisP* is involved in the extracellular processing of a fully matured precursor nisin; *nisR* and *K* encode regulatory proteins involved in gene expression and *nisI* is involved in immunity to nisin. The nucleotide sequence of the *nisABTCIPRK* gene cluster is shown in Figures 7 and 8.

Preferably the variant *nisA* gene occupies the same position as the natural *nisA* gene in the gene cluster. It is preferred if the cell is a lactococcal cell, most preferably the cell is a *Lactococcus lactis* cell. Suitable cells, especially Lactococcus cells, are readily available to the skilled person. Clearly it is required that they are a nisin producing cell, preferably a nisin producing, maturing and secreting cell but any such cells can be used. For example, the naturally-occurring nisin-producing strain NCFB894 as deposited in the National Collection of Food Bacteria at the Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney, Norwich NR4 7UA, UK (and as described in Gasson (1984)

FEMS Microbiol. Lett. 21, 7-10) is a suitable Lactococcal cell for use in the methods of the invention.

By "natural *nisA* nisin" we include a peptide antibiotic produced by some naturally occurring nisin-producing strains of bacteria. The mature molecule is based on a sequence of amino acids encoded by a gene, *nisA*. The chemical structure of a natural *nisA* nisin is shown in Figure 1. We also include in the term "natural *nisA* nisin" other naturally-occurring nisins that are based on, but vary from, the *nisA* nisin shown in Figure 1. For example, we include nisin Z which has the same chemical structure as the *nisA* nisin shown in Figure 1 except histidine in position 27 has been replaced by asparagine. The gene which encodes nisin Z was found to contain only one nucleotide substitution in comparison with the *nisA* gene which encodes the nisin A shown in Figure 1.

By "elevated level of its natural *nisA* nisin compared to the natural level" we include a cell modified according to the method which produces at least 5% more, preferably 10% more, more preferably 50% more and most preferably > 100% more natural *nisA* nisin than an unmodified cell when grown under the same culture conditions.

By "variant nisin" we include a protein-engineered variant of a natural *nisA* nisin in which changes to the amino acid sequence have been made as a result of site-directed or random mutagenesis of a *nisA* gene. Conveniently, one or more missense mutations are introduced into the protein coding region which result in one or more amino acids being substituted for another. Alternatively, a nonsense mutation can be introduced such that a truncated nisin is produced. In this case, the nisin still retains antibi tic activity. As a further alternative, deletions and/or insertions of the *nisA* gene can be made so long as the resulting nisin still

retains antibi tic activity.

Site-directed mutations of the *nisA* gene may be made, for example, by the oligonucleotide-directed mutagenesis technique of Zoller & Smith (1983) *Meth. Enzymol.* **100**, 468-500 and Zoller & Smith (1984) *DNA* **3**, 479-480 which uses mismatched oligonucleotide primers to introduce the mutation. It is convenient to use a method for improving the yield of mutants, for example, the *dut-ung* method described by Kunkel (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488-492. Alternatively, the polymerase chain reaction (PCR) may be used to generate mutants using mismatched oligonucleotides (Saiki *et al* (1988) *Science* **239**, 487-491). Random mutants of the *nisA* gene can be made chemically using, for example, sodium bisulphite or hydroxylamine as the mutagen. Alternatively, random mutations can be introduced into the *nisA* gene using enzymatic misincorporation using a DNA polymerase with relatively low fidelity, for example AMV reverse transcriptase or *Taq* DNA polymerase or by using mixtures of oligonucleotides, spiked during synthesis, to incorporate a small amount of each different bases at each position. These methods are well known in the art.

By "variant *nisA* gene" we include fragments of a *nisA* gene wherein the said fragments vary when compared to the equivalent part of the natural *nisA* gene.

By "variant *nisA* gene" we do specifically include genes in which the promoter region of the natural *nisA* gene is replaced by another (heterologous) promoter, preferably one which is known to be a more powerful pr moter than the natural *nisA* gene promoter. Examples of suitable promoters are the inducible *lacA* promoter (van Rooijen *et al* (1992) *J. Bacteriol.* **179**, 2273-2280) and the T7 promoter (Wells *et al*

(1993) *Mol. Microbiol.* 5, 1155-1162), both papers being incorporated herein by reference.

We also include in the term "variant *nisA* gene" genes in which the ribosome binding region of the natural *nisA* gene is modified, preferably to improve the efficiency of initiation of translation of the *nisA* coding region.

Also included in the term "variant *nisA* gene" are genes which have silent mutations in the coding region, that is genes in which one or more codons are changed for their synonym, but that the natural *nisA* nisin is encoded thereby. Efficiency of translation may be improved by using such variant nisin coding regions. We also include genes which comprise a heterologous promoter to drive transcription of a variant coding region, that is, a promoter other than the natural *nisA* gene promoter.

In all cases, it is preferred that the combination of promoter, ribosome binding site and coding region gives optimal expression of the nisin encoded by the coding region.

Variant nisins which have improved properties compared with natural *nisA* nisin are preferred, for example those variant nisins which have more potent antimicrobial activity or that have greater resistance to hydrolysis or degradation when added to foodstuffs. Variant nisins are described in WO 93/20213 and WO 92/18633 (incorporated herein by reference), and in the Examples that illustrate the present invention.

A preferred embodiment of the invention provides a method for making a cell which either (a) does not express its natural *nisA* nisin but expresses

a variant nisin or (b) expresses an elevated level f its natural *nisA* nisin compared to the natural level and, in either case, is capable of expressing genes for nisin modification and immunity comprising the step of substituting a variant *nisA* gene or part thereof for the natural, chromosomal *nisA* gene or part thereof at the chromosomal location of the said natural *nisA* gene.

The variant *nisA* gene or part thereof can be substituted for the natural, chromosomal *nisA* gene or part thereof at the chromosomal location of the said natural *nisA* gene in one step by gene replacement. Conveniently, a plasmid containing the variant *nisA* gene or part thereof is introduced into a host cell containing a chromosomal copy of the natural *nisA* gene (and preferably the genes for nisin modification, immunity and translocation of nisin out of the cell). A double cross-over recombination event can lead to the natural *nisA* gene or part thereof being replaced by the variant *nisA* gene or part thereof. The resulting cell will contain a chromosomal copy of the variant *nisA* gene and hence produce variant nisin provided that the variant *nisA* gene comprises a coding region which has been modified.

It is not necessary that the whole of the *nisA* gene is replaced. Rather, it is convenient that a or the part of the *nisA* gene that encodes the amino acid changes present in the variant nisin or contains the heterologous promoter is replaced.

The *nisA* gene, and other genes necessary for nisin biosynthesis, maturation and secretion are, in nature, located on a transposon which is part of the chromosome. Thus, chromosomal location refers to the presence of the *nisA* gene in the chromosomal DNA within the nisin gene cluster (*nisABTCIPRK*) rather than the position of the gene cluster relative to other genetic markers on the chromosome.

It is well known that hom l gous recombination occurs very inefficiently and unpredictably in Lactococcus and, although the above described direct, one-step method is feasible, it is more preferred if the gene replacement is carried out in an indirect, two step process in which it is possible to select for the desired recombinants as now described:

A further preferred method comprises the steps of (1) substituting a counter-selectable *nisA* gene or part thereof for the natural, chromosomal *nisA* gene or part thereof at the chromosomal location of the said natural *nisA* gene and (2) substituting a variant *nisA* gene or part thereof for the counter-selectable *nisA* gene or part thereof at the chromosomal location of the said natural *nisA* gene.

By "counter-selectable *nisA* gene" we include a *nisA* gene modified so that it is readily distinguishable from either the natural *nisA* gene or from a variant *nisA* gene.

Conveniently, the counter-selectable *nisA* gene is a *nisA* gene in which an antibiotic resistance gene (such as that for erythromycin resistance) has been inserted or is a *nisA* gene in which some or all of the coding region has been deleted. It is preferred, but not necessary, that the counter-selectable *nisA* gene does not express nisin.

It is not necessary that the whole of the *nisA* gene is replaced. Rather, it is convenient that a or the part of the *nisA* gene containing the counter-selectable marker is replaced.

In these examples the counter-selectable *nisA* gene can be distinguished from the natural r variant *nisA* gene by resistance to antibiotic of the counter-selectable gene and/or by size differences.

Thus, it is relatively straightforward to determine whether step 1 of the preferred method has been achieved because the resulting cell will, for example, have gained antibiotic resistance or, if the counter-selectable *nisA* gene has a deletion, specific fragments of the cell's chromosomal DNA will be missing or reduced in size.

Whether a specific fragment of a cell's chromosomal DNA is missing or reduced in size can readily be determined using well known molecular techniques such as Southern blotting, polymerase chain reaction (PCR) analysis or restriction fragment length polymorphism (RFLP) analysis.

Similarly, it is relatively straightforward to determine whether step 2 of the preferred method has been achieved because the resulting cell will, for example have lost antibiotic resistance or gained a fragment of chromosomal DNA.

Conveniently, in this preferred embodiment, there is a selection associated with step 2. For example, it is preferred if the counter-selectable gene in step 1 comprises a deletion of all or part of the *nisA* coding region (Δ*nisA*) and that in step 2 the correct replacement of the variant *nisA* gene is selected for. Thus, in a preferred method, a *Lactococcus lactis* strain, containing a Δ*nisA* gene (made using step 1) is used in step 2. A thermosensitive shuttle vector (replication-permissive at low temperature but not at high temperature) is used to introduce the variant *nisA* gene into the chromosome of the Δ*nisA* strain. For example, the Δ*nisA* strain is transformed with a plasmid containing the variant *nisA* gene and a gene for antibiotic resistance, and the cell is incubated at the permissive temperature in the presence of antibiotic. The cell is then transferred to the non-permissive temperature in the presence of antibiotic and a single cross-over vent results in the integration of the plasmid in the

chromosome at the site f plasmid/chromosome hom logy (ie at the common regions of the Δ*nisA* and variant *nisA* gene).

The cell is then transferred to the permissive temperature to allow plasmid replication. Recombination between homologous sequences flanking the integrated plasmid results in its excision from the chromosome. A second cross-over event occurs resulting in either sequences originating from the integrated plasmid (ie the variant *nisA* gene) or the original sequences (ie the counter-selectable *nisA* gene) being retained on the chromosome. As discussed above, the variant *nisA* gene and counter-selectable *nisA* gene can be distinguished, and cells containing the variant *nisA* gene are chosen.

Cells are cured of plasmid by culturing in the absence of antibiotic.

In this preferred method the entire *nisA* gene and flanking sequences are effectively replaced with the identical sequences, with the exception of the specifically incorporated mutation. The size of the plasmid DNA fragment, containing the variant *nisA* gene is limited by the requirement for homologous sequences (on both the plasmid and chromosome) across which recombination can take place to bring about plasmid integration and subsequently, gene replacement. It is preferred for vector construction that there is approximately 1 kb of homology flanking the site of any sequence alteration. As an example our gene replacement vector has approximately 800 bp on one side of the *nisA* gene and 1,200 bp on the other. A reduction in this size would be expected to reduce the incidence of homologous recombination and therefore the chances of detecting the desired gene replacement. Figure 10 illustrates the recombinati n events which occur during the preferred method.

The method of the inventi n results in cells which produce a variant nisin from a chromosomal copy of a variant *nisA* gene or a natural *nisA* nisin from a chromosomal copy of a variant *nisA* gene. As has been discussed above, it is most preferred if variant nisins have antibiotic activity. Thus, the cells will exhibit a Nis^+ phenotype because the cells produce a nisin (either natural or variant).

We have determined that these nisin-producing cells must necessarily also be immune to the nisin at the level at which they produce this antimicrobial peptide. Thus, a further preferred embodiment of the method comprises a further step of selecting those cells which are immune to nisin, at least to a level of 1000 U/ml.

Although it is preferred that the cells produced by the method express a variant nisin, the method also encompasses the making of a cell which can express natural *nisA* at a high level from a powerful, heterologous promoter.

In a less preferred embodiment, the gene cluster comprises a variant *nisA* gene and the genes for nisin modification and immunity and this gene cluster is carried on an autonomously replicating DNA element. Conveniently, the autonomously replicating DNA element is a plasmid. The host cell for the plasmid is a cell that does not express a natural *nisA* nisin. For example, a Lactococcal cell in which the natural nisin genes are absent or the natural *nisA* gene is inactivated.

Cloning the entire nisin gene cluster on a plasmid involves the integration of a large segment ($\sim$11 kb) of DNA. A strategy of this type has the advantage of enabling the copy number and therefore gene dosage to be altered and also may facilitate the transfer of nisin determinants to a range

of alternative host backgrounds. There are two preferred types of replicons (which use different modes of replication) which can be employed as suitable vectors:- the rolling circle plasmids (for example pTG262, Dodd *et al* (1990) *J. Gen. Microbiol.* **136**, 555-566) or the theta type plasmids (for example, pIL253, high copy number, and pIL277, low copy number, Simon & Chopin (1988) *Biochimie* **70**, 559-566). Both papers are incorporated herein by reference. When the method of the invention uses a lactococcal cell it is preferred if the plasmid is a shuttle plasmid, that is a plasmid that can replicate in the lactococcal cell and can also replicate in another host cell such as *Escherichia coli*.

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3′-single-stranded termini with their 3′-5′-exonucleolytic activities, and fill in recessed 3′-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as

bacteriophage T4 DNA ligase. Thus, the products f the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

A second aspect of the invention provides a cell which does not contain a natural *nisA* gene but expresses a nisin comprising a variant *nisA* gene wherein the variant *nisA* gene has the same relationship as the natural *nisA* gene to a gene cluster containing the natural *nisA* gene and the genes for nisin modification, secretion and immunity.

The cell of the second aspect of the invention is obtainable by the methods described in the first aspect of the invention.

In the most preferred embodiment the natural, chromosomal *nisA* gene or part thereof is absent and the cell comprises a variant *nisA* gene or part thereof at the chromosomal location of the said natural *nisA* gene.

Preferably the cell is a Lactococcus, most preferably *Lactococcus lactis*.

It is preferred that the cell expresses a variant nisin, although a cell that expresses an elevated level of natural *nisA* nisin also forms part of the invention.

Conveniently the variant *nisA* gene contains transcriptional or translational control sequences which enable the cell to either express a variant nisin or, in the case of natural *nisA* nisin, enable the cell to express it at an elevated level. Thus, in one embodiment the cell comprises a variant *nisA* gene consisting of a heterologous promoter which drives the expression of a nisin coding region (which may express a natural *nisA* nisin or a variant nisin).

In a less preferred embodiment the cell comprises an autonomously replicating DNA element carrying a variant *nisA* gene and the genes for nisin modification and immunity. In this case, the cell does not have an active chromosomal *nisA* gene and preferably no chromosomal nisin genes.

A third aspect of the invention provides a process for producing nisin comprising culturing a cell as described in the third aspect of the invention and obtaining the nisin produced thereby.

Conveniently, the nisin is a variant nisin.

It is preferred if the cells are those of the most preferred embodiment.

We have found that using the cells of the most preferred embodiment in the process we can produce an unexpectedly high yield of nisin particularly in comparison to the known processes which rely on plasmid-borne *nisA* genes to express the nisin (in the absence of plasmid-borne nisin immunity, modification and secretion genes). Further details of this surprising effect are given in the Examples. However, it is worth noting at this point that, in the case of a nisin variant in which both dehydroalanine 5 was replaced by alanine and dehydroalanine 33 was replaced by alanine (known as nisinA/Dha 5A, Dha 33A), the cell of the present invention in which the natural *nisA* gene is replaced by the variant *nisA* gene produces more than 100 times the nisin compared with a prior art cell in which the same variant nisin (nisinA/Dha 5A, Dha 33A) was encoded by a plasmid. In addition, all of the variant nisins that have been tested give a higher yield from cells of the present invention compared with the prior art cells containing the variant nisin gene on a plasmid.

Further advantages over the prior art methods and cells are obtained using the cells of the most preferred embodiment to produce nisin. For example, because the cells do not contain a plasmid there is no requirement for antibiotic selection during their culture and plasmid loss during culture is not a problem.

Thus, for stability there is an advantage in that the variant nisin gene is integrated within the bacterial chromosome albeit as part of the nisin transposon Tn*5301*. The latter is extremely stable and we have in fact found it difficult to eliminate deliberately. In the laboratory selection for a plasmid marker prevents this being a practical problem, but for industrial use this would be a disadvantage. It may be undesirabl to add

antibiotics to the fermentati n.

In a less preferred embodiment, cells carrying the variant *nisA* gene and the genes for nisin modification and immunity are carried on an autonomously replicating DNA element, such as a plasmid. Clearly, in this embodiment, plasmid selection is required during the culture of the cell.

A particularly preferred embodiment of the third aspect of the invention is wherein the cells are cultured in the presence of *nisA* nisin or a variant nisin which can induce nisin expression. Nisin A wherein Ile30 is replaced by Trp (I30W) is an example of a variant that, as a result of its mutation, does not function well as an inducer of its own biosynthesis. By adding sub-inhibitory concentrations of nisin to the growth medium, during fermentation, higher levels of the variant nisin are produced. Any variants that are less efficient as inducing agents benefit from the inclusion of nisin in the growth medium (ie a nisin induction step in the purification procedure). The amount of induction varies depending on the initial induction capacity of any particular variant (with I30W nisin A production more than doubled as a result of induction). Induction may be routinely included in the method as a means of maximising production levels. Any concerns about contamination with the wild type molecule are minimal as the nisin concentration required for induction is negligible compared to the amount of nisin variant being purified. Conveniently, the *nisA* nisin is a minimum amount that provides maximal induction of nisin production. This amount can be determined empirically by a person skilled in the art. Suitable *nisA* nisin concentrations for induction in this embodiment are from 1 nM to 500 nM, preferably 10 nM to 250 nM, more preferably 50 nM t 150 nM, most preferably 100 nM.

A reverse-phase HPLC step in any purification would ensure separati n of any residual *nisA* nisin from variant nisins.

A fourth aspect of the invention provides a nisin produced by the process of the invention.

The presence of unsaturated amino acids in lantibiotics including nisin and the role they play in the biological properties of these complex molecules is of particular interest in structure/function analyses. It has been proposed that the reactive unsaturated bonds that characterise dehydro-amino acids play a functional role in the antimicrobial activity that subtilin, a lantibiotic, exerts against bacterial spore outgrowth. These residues have also attracted attention as a possible source of molecular instability. It has long been known that the antimicrobial activity of commercial samples of natural *nisA* nisin deteriorate on storage and that a number of chemical components are found within such samples (Berridge *et al*, 1952) and Chan *et al* (1989) have demonstrated that specific cleavage occurs at the dehydroalanine residues in the mature molecule. Cleavage at Dha5 results in the opening of the first lanthionine ring of nisin and is accompanied by a loss of antimicrobial activity. In contrast, the degradation product arising as a result of cleavage at Dha33 retains essentially wild type activity (Chan *et al*, 1989).

In WO 93/20213 we described the construction of *L. lactis* derivatives expressing nisinA/Dha5A, nisinA/Dha33A and nisinA/Dha5A,Dha33A. We also demonstrated in that work that these engineered nisins retained their antimicrobial activity against sensitive indicator strains. Clearly, as described in detail in the Examples, these nisins can be produced more efficiently by the present process. However, the present process can also be used to produce any further variant nisins which have other, improved

properties so long as they are encoded by a variant *nisA* gene.

A fifth aspect of the invention provides the use of a nisin produced according to the process of the invention as an antimicrobial agent. The ability of nisin to inhibit growth of spoilage bacteria and food pathogens has resulted in the extensive use of as a natural preservative in certain food products, particularly dairy products such as soft cheeses. Variant nisins are also used.

The invention will now be described in more detail with reference to the following figures and examples wherein:

Figure 1 shows the molecular structure of natural nisinA. Changes that have been made to the sequence as a result of protein engineering are indicated by arrows.

Figure 2 shows diagrammatically some of the recombinant plasmids constructed and used in this work.

Figure 3 shows diagrammatically counter-selectable *nisA* genes wherein either an erythromycin resistance gene is inserted in a *nisA* gene or a frame-shift deletion has been made (*nisA*-fs). The sequences shown are in the sequence listing as SEQ ID Nos. 12 to 17.

Figure 4 shows the nucleotide sequence of at least part of the natural *nisA* gene and expression signals showing changes introduced by PCR-mediated site-specific mutagenesis. *Bam*HI and *Bgl*II sites flanking *nisA* were engineered into plasmid pFI740 (Figure 2d). The substitution of Ser5 and Ser33 codons for alanine cod ns in variant *nisA* genes (Table 1) is shown above the sequence. The sequences shown are in the sequence listing as

SEQ ID Nos. 18 and 19.

Figure 5 shows an agarose gel electrophoresis of PCR fragment generated with primers P39 and P40. PCR reactions were carried out on colonies of:- track 3, FI5876; 4, FI7990; 5-10, FI7990 (pG host6 derivative) after gene replacement procedure. Size standards:- tracks 1 and 12, λDNA digested with *Bgl*I; 2 and 11, λDNA digested with *Hind*III.

Figure 6 shows a plate diffusion bioassay. 150 μl samples of cell free extracts from strains:- 4, FI5876; 5, FI7990; 6, FI8070; 7, FI8198; 8, FI8199 were loaded into wells bored in MRS agar seeded with the indicator strain *Lactobacillus helveticus* CH-1. Plates were incubated overnight at 42°C. Standards included on the assay plate are:- 1,50; 2,100; 3,200; 9,300; 10,400 U/ml.

Figure 7 shows sequences of the *nisA*, *nisB*, *nisT*, *nisC* and *nisI* genes of Tn5276 of *L. lactis* NIZO R5, and is taken from Kuipers *et al* (1993) *Eur. J. Biochem.* **216**, 281-291. Putative ribosome-binding sites (RBS) and inverted repeats (→) are indicated, as is the transcription-initiation site of the *nisA* gene and its preceding canonical sequences. Positions of restriction sites used are as follows: *Acc*I, 6383-6388; *Bcl*I, 2914-2919; *Eco*RI, 3461-3466; *Eco*RV, 1805-1810; *Hae*III, 6509-6512; *Nco*I, 6218-6223; *Nde*I, 4518-4523; *Pst*I, 7418-7423; *Sst*I 283-288, 1547-1552 and 2463-2468.

Figure 8 shows a nucleotide sequence of cloned 5.0-kb region downstream from *nisC* with open reading frames *nisI*, *nisP*, *nisR*, and *nisK*, and is taken from Engelke *et al* (1994) *Appl. Environ. Microbiol.* **60**, 814-825. Possible ribosome-binding sites (RBS), restriction sites, and inverted repeats are underlined. Open reading frames are designated by a ne-

letter code. Arrows indicate the putative signal peptide cleavage sites f *NisI* and *NisP*; the putative membrane anchor sequence of *NisP* is underlined. Conserved, functional, and active-site amino acids are written in boldface letters and marked by asterisks.

Figure 9 illustrates a gene replacement vector. Sizes of the cloned fragments that make up the *nisA* cassette and flanking sequences are given in base pairs.

Figure 10 describes diagrammatically a gene replacement protocol.

Figure 11 shows the double-stranded nucleotide sequence of *nisA* gene and pre-nisin amino acid sequence. The -35 and -10 regions and the transcription initiation site are indicated together with restriction enzyme sites used in the *nisA* gene cassette (see Figure 2) above the DNA sequence. The location of primers (5'-end) employed in amplification of the cassette fragments and PCR-mediated mutagenesis are shown, above and below the sequence, as horizontal black arrows indicating the direction of DNA synthesis. Specific amino acid substitutions, as a result of the mutagenesis, are shown below the pre-nisin sequence.

Figure 12 is a representation of the organisation of the *nis* genes.

Example 1: Construction of Lactococcal cells in which the natural, chromosomal *nisA* gene is replaced by a variant *nisA* gene

Methods

Microbi logical techniques and strains used. The Lactococcal strains used in this study and their derivation are given in Table 1.

Table 1. Lactococcal strains used in this study:-

Strain	*nisA* mutation	Activity	Immunity (U/ml x 10^3)	Reference
MG1614	-	-	0.01	Gasson (1983) *J. Bacteriol.* 154, 1-9
FI5876	wild type	+	>1	Dodd *et al* (1990) *J. Gen. Microbiol.* 136, 555-566; Horn *et al* (1991) *Mol. Gen. Genet.* 228, 129-135
FI7847	*nisA*-(fs)	-	0.5-0.75	This work
FI7990	Δ*nisA*	-	0.25-0.5	This work
FI8070	*nisA*/S5A	+	>1	This work
FI8198	*nisA*/S33A	+	>1	This work
FI8199	*nisA*/S5A,S33A	+	>1	This work
FI7893	*nisA*	+	>1	This work
FI8003	*nisA*	-	0.25-0.5	This work

Unless stated otherwise, cultures were grown at 30°C in M17 medium (Terzaghi & Sandine (1975) *Appl. Environ. Microbiol.* 29, 807-813) supplemented with 0.5% (wt/vol) glucose (GM17 medium). Screening strains for resistance to antibiotics was carried out at the following levels: erythromycin, (Em^r) 5 μg/ml; streptomycin, (Sm^r) 200 μg/ml.

Escherichia coli MC1022 (Casadaban & Cohen (1980) *J. Mol. Biol.* 138, 179-207 was the host strain for construction and molecular analysis of recombinant plasmids derived from the vectors pMTL23p (Chambers *et*

al (1988) *Gene* 68, 139-149), pGEM-3Z (Promega), pCR™II (Invitrogen) and pG+host6 (Appligene). Recombinant plasmids used, and constructed during the course of this study, are shown in Figure 2. *E. coli* cultures were propagated at 37°C in L broth (Lennox (1955) *Virology* 9, 190-206. Selection for ampicillin resistance (Ap^r) was carried out at 100 μg/ml, chloramphenicol (Cm^r) at 15 μg/ml and erythromycin, (Em^r) at 400 μg/ml.

Nisin activity in Lactococcal strains was assayed by both deferred and direct means. Plate diffusion bioassays were performed as previously described (Dodd *et al* (1992) *Appl. Environ. Microbiol.* 58, 3683-3693. Colonies growing on the surface of a GM17 plate were directly assayed by inverting over chloroform for 12 minutes and overlaying with agar seeded with the nisin sensitive *L. lactis* strain MG1614. Plates were incubated overnight and zone sizes around colonies compared with those of controls. Nisin immunity was determined by streaking cultures on a series of GM17 agar plates containing an increasing concentration of nisin and assessing the degree of growth at the different nisin levels. Control cultures (FI5876, positive) and MG1614 (negative) were included on each plate.

Molecular techniques.

Total DNA, plasmid DNA was carried out as described by Dodd *et al* (1990) *J. Gen. Microbiol.* 136, 555-566 and Horn *et al* (1991) *Mol. Gen. Genet.* 228, 129-135. Restriction enzyme and other DNA modifying enzymes from various sources were used according to the suppliers recommendations. Recombinant plasmids were recovered by transformation of *E. coli* as described previously (Dodd *et al* (1992) *supra* or electroporati n f *L. lactis* according to H lo and Nes (1989) *Appl. Environ. Microbiol.* 55, 3119-2123 with the modifications of Dodd *et al*

(1992) *supra*. Conditi ns used for polymerase chain reacti n (PCR) were as described in Horn *et al* (1991) *Mol. Gen. Genet.* 228, 129-135. Primers were synthesised on an Applied Biosystems DNA synthesizer (model 381A) and are listed in Table 2. Fragments generated for the construction of gene-replacement vectors were amplified using Dynozyme (Flowgen) and cloned into pCR™II prior to nucleotide sequence confirmation. For routine PCR screening of recombinant clones AmpliTaq-DNA polymerase (Perkin Elmer) was used. Direct nucleotide sequence determination of purified PCR-generated templates was carried out on an Applied Biosystems DNA Sequencer (model 373A) using the manufacturers' Taq "Dyedeoxy" terminator cycle sequencing kit.

Table 2. Primers used in this study:-

P13 (SEQ ID No 1) 5'-AACGGATCCGATTAAATTCTGAAGTTTG-3'
*Bam*HI

P17 (SEQ ID No 2) 5'-TCAGAGCTCCTGTTTTACAACCGGGTGTACATA GTGCAAT-3'

P18 (SEQ ID No 3) 5'-TAGTATTCACGTAGCTAAATAACC-3'

P19 (SEQ ID No 4) 5'-TTGGTTATTTAGCTACGTGAATAC-3'

P25 (SEQ ID No 6) 5'-AATCGGATCCGTTTATTATGCTCGC-3'
*Bam*HI

P26 (SEQ ID No 6) 5'-ATAGTTGACGAATATTTAATAATTTT-3'
*Hinc*II

P27 (SEQ ID No 7) 5'-CTTGGTCGACACCATATTTT-3'
*Sal*I

P28 (SEQ ID No 8) 5'-GTTAGATCTGACATGGATAC-3'
*Bgl*II

P32 (SEQ ID No 9) 5'-CCATGTCAGATCTAACAAAATAC-3'
*Bgl*II

P39 (SEQ ID No 10) 5'-GACTTTCCATTATGCTTGGATTTTT-3'

P40 (SEQ ID No 11) 5'-GCTCCTATGCCAAATGTAGAATC-3'

Construction of *nisA* gene replacement vectors.

The thermosensitive shuttle vector pG+host6 was employed for carrying out gene replacement. Homologous plasmid DNA originated from pFI172 (Dodd *et al* (1990) *supra*) which contains a 2.1kb region of the FI5876 chromosome (Fig 2a) including the *nisA* gene. The entire fragment was subcloned into pG+host6 to generate the *nisA* gene-replacement vector pFI690 (Fig 2b). Subsequent manipulation of this region, resulting in inactivation or mutagenesis of the *nisA* gene (see below), was carried out in either vector pGEM-3Z or pMTL23P. The final step in the construction of each gene-replacement vector was cloning the modified 2.1kb fragment into pG+host6. The derivatives of pG+host6 were established in *E. coli* and plasmid DNA, from this host, used to transform *L. lactis* FI7990 (Table 1).

***nisA* frame shift mutation - *nisA*-(fs):-** Insertional inactivation of the *nisA* gene, by cloning an Em^r gene into the internal *Sac*I site, has been described previously (Dodd *et al* (1992) *supra*). In this plasmid the Em^r gene is flanked by a short multiple cloning site (Fig 3). Digestion with the restriction enzyme *Sma*I, followed by ligation to recircularise the vector sequences, resulted in deletion of the Em^r gene. Residual sequences from the multiple cloning site leave a 20bp insertion within the *Sac*I site and cause a frameshift mutation to occur in codon 16 of the *nisA* gene. The first 38 amino acids encoded by this mutated gene [designated *nisA*-(fs)] are unaffected. However, the predicted translation product would be a truncated prenisin (45 residues) including the on-nisin amino acid sequence RYPGTEL at its COOH-terminus (Fig 3). The *nisA*-(fs) mutation was subcloned into pG+host6 to generate the gene-replacement vector pFI674 (Fig 2c).

***nisA* deletion - Δ*nisA*:-** - Inactivation of the *nisA* gene was also achieved by deleti n f the coding region. In order to confine the deletion to just

nisA it was necessary to engineer additional restriction enzyme sites on either side of the gene. Primers were designed, for PCR amplification of this region of the chromosome, that incorporated a *Bam*HI site (P13, Table 2) 80bp upstream of the start of *nisA* and a *Bgl*II site (P32, Table 2) 25bp beyond the stop codon, as a result of 2bp changes in each case (Fig 3). The flanking fragments (shown in Fig 2d) were also generated using PCR amplification. Primers P26 and P25 were employed for amplification of the upstream 211 bp *Hinc*II/*Bam*HI fragment and primers P28 and P27 employed for the downstream 1.1kb *Bgl*II/*Sal*I fragment (Table 2). The template used for these PCR reactions was pFI172 DNA. The resulting plasmid (pFI740) contained an intact *nisA* gene flanked by an engineered *Bam*HI and *Bgl*II sites, all contained within 2.1 kb of sequences homologous to the chromosome (Figure 2d). Digestion of pFI740 with these two enzymes, followed by ligation of their compatible ends, resulted in the generation of plasmid pFI751 in which the *nisA* gene has been deleted, designated as Δ*nisA* (Fig 2e). PCR amplification of this part of the plasmid and nucleotide sequence analysis of the region spanning the deletion in the amplified fragment confirmed that fusion of the *Bam*HI and *Bgl*II sites had occurred.

***nisA* site-specific mutations:-** The construction of the plasmid pFI877 (Fig 2f) allowed a cassette mutagenesis strategy to be employed for the introduction of site-specific mutations into the *nisA* gene. This pGEM-3Z derivative contains the equivalent sequences to those in pFI690 (Fig 2b), but includes the engineered *Bgl*II site downstream of *nisA* in pFI740 (Fig 2d). In pFI877 a *Hinc*II/*Sac*I fragment encoding the amino-terminal region of *nisA* and upstream expression signals, replaced the PCR-generated fragment of pFI740 that contains the engineered *Bam*HI site. Thus, the only difference between sequences in pFI877 and the equivalent chromosomal wild-type sequences is the presence of an additi nal *Bgl*II

site downstream of the *nisA* gene. The constructi n of this *nisA* cassette is such that site-specific mutations could be readily incorporated into the gene. PCR-mediated mutagenesis was used to amplify either the *Hinc*II/*Sac*I or *Sac*I/*Bgl*II fragments containing the amino-or COOH-terminal regions of the *nisA* gene respectively (Fig 2f). These fragments, containing a specific mutation, were then substituted for the wild-type fragment of pFI877. Mutations were incorporated in either the primers used to amplify the cassette fragments or, if the desired site of mutation was internal, the technique of spliced overlap extension was used (Ho *et al* (1989) *Gene* 77, 51-59) with the specific mutations incorporated on two complementary primers spanning the mutation site (Dodd *et al* (1992) *supra*).

Gene replacement protocol.

L. lactis FI7990 transformants containing derivatives of pG+host6 were established at 28°C and grown overnight at this temperature in GM17 containing Em at 5 μg/ml (GM17-Em). Approximately 10^5 cells were used to inoculate 100 ml of fresh, prewarmed GM17-Em and the cultures were incubated at 28°C for 4 hours. Incubation was continued overnight at the elevated temperature of 37°C. This temperature is non-permissive for pG+host 6 replication (Biswas *et al* (1993) *J. Bacteriol.* 175, 3628-3635) and the presence of Em in the growth media ensures selection for those cell lines in which a single cross-over results in the integration of the derivative in the chromosome at the site of plasmid/chromosome homology (Leenhouts *et al* (1989) *Appl. Environ. Microbiol.* 55, 394-400; Leenhouts *et al* (1990) *Appl. Environ. Microbiol.* 56, 2726-2735; Chopin *et al* (1989) *Appl. Environ. Microbiol.* 55, 1769-1774). Prewarmed GM17 (with no Em) was inoculated with approximately 10^5 cells from the overnight culture and incubated overnight at 28°C. At this temperature

plasmid replication is possible and recombination between homologous sequences flanking the integrated plasmid results in its excision from the chromosome. Depending on where the second cross-over occurs either sequences originating from the integrated plasmid, or the original sequences will be retained in the chromosome. The lengths of DNA homology are shown in Figure 9. These sequences originated as an *AccI*/*SalI* fragment making up part of a *SalI* fragment that is cloned and sequenced in the plasmid pFI 172 (Dodd *et al* (1990) *J. Gen. Microbiol.* **136**, 555-566).

Cultures were diluted and spread, for single colonies, on GM17 agar plates. In order to cure the cells of plasmid, the plates were incubated at 37°C. Colonies (approximately 50) were screened for loss of the pG+host6 derivative by patching onto GM17 plates containing Em (5 μg/ml). When using this technique to disrupt the *nisA* gene colonies were also screened for loss of nisin activity and PCR analysis of the relevant region of the chromosome used to confirm any changes at the molecular level.

The gene replacement protocol is illustrated diagrammatically in Figure 10.

RESULTS

Inactivation of chromosomally encoded FI5876 *nisA* gene.

In order to identify cell lines that have acquired variant *nisA* genes it was convenient to first construct a Nis⁻ host strain, by inactivating the resident *nisA* gene. The well characterised nisin-producing strain *L. lactis* FI5876 was selected for this purpose (Dodd *et al* (1990) *supra*; Horn *et al* (1991)

supra). The nisin biosynthesis genes from this strain have been cloned and sequenced (Dodd *et al* (1992) *supra*).

Gene-replacement was used to substitute the wild-type *nisA* gene of FI5876 with the plasmid pFI674-encoded *nisA*-(fs) gene (Fig 2c, Fig 3). Of fifty colonies screened five were both Em^s and Nis^- suggesting that in these FI5876 derivatives gene replacement had occurred. Furthermore, this result indicated that the modified *nisA* gene, was defective and did not express a precursor molecule that could be matured to an active form. One of the Nis^- strains, designated FI7847, was analysed further. To test the system it was demonstrated that the *nisA*-(fs) mutation in FI7847 could be reverted back to wild-type by carrying out the equivalent experiment using the *nisA* gene replacement vector pFI690 (Fig 2b). Recovery of nisin production by the resulting gene-replaced strain, FI7898, indicated that the Nis^- phenotype exhibited by FI7847 was due solely to disruption of the *nisA* gene. The other nisin biosynthesis determinants appeared to have been unaffected by the switching of *nisA* genes.

An alternative approach was to generate a Nis^- host by deletion of the entire chromosomal *nisA* gene in FI5876. Plasmid pFI751 (Fig 2e) was constructed for this purpose and gene replacement used to incorporate the approximate 300 bp deletion Δ*nisA* (Fig 4) in place of *nisA*. Nis^- strains were recovered at about the same frequency as was found with the *nisA*-(fs) gene-replacement. In this case the Δ*nisA* containing strains could be readily distinguished from the parent strain by PCR analysis. Primers P39 and P40 (Table 2, Fig 2a) amplified a 1.8kb fragment in the gene-replaced strains (Fig 5, track 4) compared to a 2.1kb fragment generated from the equivalent region of FI5876, encoding wild-type *nisA* (Fig 5, track 3). In one of the Nis^- strains, designated FI7990, nucleotide sequence analysis of the PCR-generated 1.8kb fragment confirmed that Δ*nisA* was

incorporated in the correct region of the chromosome. Again the system was tested and it was shown that nisin production could be restored in a FI7990 derived strain by gene replacement with pFI690 (Fig 2b). PCR analysis of these Nis$^+$ colonies demonstrated that the Δ*nisA* mutation (1.8kb fragment) had been replaced by the wild-type *nisA* gene (2.1kb fragment). As this system has the advantage of being able to readily identify gene-replacement on the basis of PCR analysis (see Fig 5) further characterisation and mutant construction was carried out using FI7990 as the host strain.

Nisin immunity

The effect of disruption of the *nisA* gene on immunity of the host strain to nisin has been described previously (Dodd *et al* (1992) *supra*). As would be expected both FI7847 [*nisA*-(fs)] and FI7990 (Δ*nisA*) displayed reduced immunity to nisin (Table 1). The *nisA* deleted strain FI7990 was sensitive to nisin at a concentration of between 250 and 500 U/ml compared to the parent strain, FI5876, which will continue growing in the presence of nisin to over 1000 U/ml. Interestingly, FI7847, encoding a truncated *nisA* gene, exhibited intermediate levels of immunity to nisin (an upper limit of between 500 and 750 U/ml with poor growth continuing to 1000 U/ml, Table 1).

A possible explanation for the difference in nisin sensitivity of these two Nis$^-$ strains came from gene replacement studies involving the vector pFI740 (Fig 2d). Strain FI8003, generated by substitution of the defective *nisA* gene with the intact plasmid pFI740-encoded *nisA*, had a Nis$^-$ phenotype. This result contrasts with that of the equivalent gene replacement experiment, involving pFI690 (Fig 2b), in which a Nis$^+$ phenotype was recovered (see above). The only difference between the

tw sequences involved is that pFI740 has an additional *Bam*HI site incorporated 80bp upstream of the ATG start codon of *nisA*, and a *Bgl*II site immediately downstream of the coding region (Fig 4). Examination of these sequences revealed that the *Bam*HI site overlaps with the proposed -35 region of the promoter identified by Kuipers *et al* (1993) *Eur. J. Biochem.* 216, 281-291. A single base pair change introduced as a result of engineering the *Bam*HI site has the effect of converting the -35 sequence from CTGATT to CCGATT (Fig 4).

These results suggest that in pFI740 the natural *nisA* promoter has been disrupted and hence, those strains, such as FI8003, which have incorporated the *Bam*HI site by gene replacement will also have acquired the defective promoter. The increased nisin sensitivity of these strains (approximately 50% that of wild-type), despite an intact *nisA* gene, suggest that these potentially promoter active sequences play a role in nisin immunity.

The preferred protocol uses nisin immunity as a means of directly selecting Nis^+ strains that have undergone gene replacement and relies on the fact that inactivation of the *nisA* promoter in FI7990 results in a sufficiently high sensitivity to nisin that the parent strain will not grow on the selective plates. Nis^- strains that retained the upstream promoter sequences (eg FI7847) were unsuitable for this procedure as they grew well at the levels of nisin that were found to be optimal for selection of Nis^+ recovery, ie 500 U/ml (Table 1).

Gene replacement - identification of variant *nisA*-encoding strains.

From the preliminary gene-replacement experiments carried out in the construction and testing of the Nis^- strains FI7847 and FI7990 it was

known that substitution of chromosomal sequences for the equivalent homologous region carried by the pG+host6 derivative, occurred at low frequency. The subsequent restoration of an intact *nisA* gene in these hosts, by gene replacement, would be expected to lead to the recovery of nisin activity. Any Nis^+ strains within the population would then be at a selective advantage over the original Nis^- parent. However, initial attempts to recover an activate *nisA* gene again resulted in the majority of colonies screened retaining the defective *nisA* parental sequences.

The restoration of a Nis^+ phenotype necessitates a functional nisin immunity mechanism and this requires the expression of the *nisA* gene. The gene-replacement protocol, employed for the construction of FI7847 and FI7990, was modified to facilitate the identification of derivatives that had acquired *nisA* or variant *nisA* genes that resulted in nisin production. The recovery of a Nis^+ colonies hinged on our interpretation that these cells must necessarily also be immune to nisin at the level at which they were producing this antimicrobial peptide. In the modified gene replacement protocol the final step included the addition of nisin to the GM17 agar plates, at a level of 500 U/ml. Nisin immune colonies that grew on this media were screened for Em^r and assayed for nisin production. PCR analysis was also used to determine the organisation of genomic sequences. Figure 5 shows the fragments generated by PCR (using primers P39 and P40, Fig 2a) from six colonies that had been through the gene replacement procedure. All were found to have acquired a functional copy of a *nisA* gene (in this case *nisA*/S5A) as shown by the 300bp increase in size of the PCR fragment. This procedure was found to be a very reliable means of identifying Nis^+ derivatives of FI7990 as this host strain was itself sensitive to the levels of nisin employed in the selection plates. The majority of colonies (approximately 90%) screened in this way were found to have undergone gene-replacement and to be

expressing a functi nal *nisA* gene r variant in place of the chromosomal lesion Δ*nisA*. This strategy has been successfully employed to select for several derivatives of FI7990 that are now exclusively expressing engineered nisins in place of nisin A.

As described above, the protocol involves the integration of the thermo-sensitive plasmid, p+Ghost6 in the chromosome, followed by its excision. Assuming that cross-overs occur with equal frequency between homologous sequences on either side of the mutation, it would be predicted that the number of cells now carrying the mutation would be the same as those cells identical to the parent strain. This did not prove to be the case and the majority of colonies screened retained the genetic organisation of the parent strain FI7990. The reason for this is not clear, but it suggests that the immediate effect of integration of a functional *nisA* gene is detrimental to the host cell. It has been reported that expression of the *nisA* gene precedes that of the adjacent *nisB* gene by 30 minutes (Engelke *et al* (1994) *supra*) and transcription of other determinants in the nisin gene cluster may be similarly delayed, with respect to prenisin production. Those strains that acquire a *nisA* gene by gene-replacement may not have recovered full immunity before the nisin molecule exerts its antimicrobial action. Such strains would not be viable. However, we have been able to restore a Nis^+ phenotype by gene replacement when nisin production has been delayed allowing full nisin immunity to be established.

Conversion of Dha to Ala residues.

The dehydroalanine (Dha) residues at positions 5 and 33 (Fig 1) were initially targeted for engineering changes in the nisin molecule. The aim was to substitute the serine residues. from which the Dhas are derived, for

alanines which lack a potentially unstable unsaturated side chain. The mutation in *nisA*,S5A was generated by PCR using primers P26 and P17 (Table 2). Amplification resulted in a 404bp *Hinc*II/*Sac*I fragment containing the amino-terminal end of *nisA* and including the substitution of a Ser codon (coordinate 173, Fig 4) for CGT which specifies alanine. The 90bp *Sac*I/*Bgl*II fragment containing the COOH-terminal end of *nisA* was generated by PCR using primers P10 and P32 (Table 2) and included a spliced overlap extension step with primers 18 and 19 (Table 2). This latter pair of complementary primers contain an alanine codon, CGT, in place of the serine codon at coordinate 257 (Fig 4). Subcloning these PCR generated fragments, either separately or together, into the appropriate gene-replacement vector resulted in an uninterrupted coding region specifying either a *nisA*/S5A, *nisA*/S33A or *nisA*/S5A,S33A gene. Transformation of FI7990 with plasmid DNA followed by the gene-replacement procedure generated a number of colonies the majority of which were found to be Em^s and Nis^+. The relevant region of the chromosome was investigated by PCR using the primer combination P39 and P40 (Fig 2a) and in each case a 300bp increase in fragment size, compared to FI7990 (see Fig 5), indicated that gene replacement had occurred. Nucleotide sequence analysis of these PCR generated fragments confirmed that, in each case, the three variant *nisA* genes were incorporated in the chromosome, in place of the Δ*nisA* lesion. A representative of each gene-replaced strain, FI8070 (*nisA*/S5A), FI8198 (*nisA*/S33A) and FI8199 (*nisA*/S5A,S33A) was characterised further.

Expression of all three mutated *nisA* genes resulted in the production of an active molecule as determined directly by colony overlays. Plate diffusion bioassays on cell extracts demonstrated that the levels of antimicrobial activity against *Lactobacillus helveticus* were comparable to that of the parent strain FI5876 (Fig 6). FI8070, encoding *nisA*/S5A,

generated a zone of inhibition similar in size to that of the parent strain FI5876. This suggests that the mutation in the nisin variant (nisinA/Dha5A) does not significantly affect its antimicrobial properties against this indicator organism. A cell extract from FI8198 (producing the variant nisin A/Dha33A) was consistently found to generate a zone of inhibition larger than that of the parent strain (Fig 6). This corresponds to an increase of approximately 50% of nisin A levels, under the conditions used here (Table 1). This higher level of production was not found in the extracts of FI8199, producing the double mutant nisin A/Dha5A,Dha33A. The inhibitory effect of this nisin variant was equivalent that of nisin A containing the single mutation and also the wild type molecule (Table 1). In all cases the yield for a particular nisin variant was higher when using this gene replacement strategy than when the equivalent plasmid-encoded gene was employed in a plasmid complementation system (Table 3, Dodd *et al* (1992) (1993) *supra*).

Table 3. Comparison of nisin activity from Lactococcal expression systems.

nisin variant	nisin activity[a] (% of wild type)	
	complementation[b]	gene replacement[c]
nisin A (wild-type)	50	100
nisin A (Dha5A)	25	100
nisin A (Dha33A)	10	150
nisin A (Dha5A,Dha33A)	<1[d]	100

[a] determined from plate diffusion bioassays

[b] antimicrobial activity achieved by plasmid-encoded *nisA* genes complementing *nisA* deficiency in host strain FI7332

[c] antimicrobial activity achieved by gene replacement. Functional

nisA gene incorporated in the chromosome of FI7990 in place of *nisA* deletion.

[d] activity was below the level of detection of the bioassay

The system developed here is producing variant nisins at yields equivalent to that of the nisin A-producing parental strain FI5876 (Table 1). In the case of the wild-type *nisA* gene, this is about 50% higher than nisin levels previously achieved using an analogous plasmid complementation approach (Dodd *et al* (1992) (1993) *supra*). A further comparison of these two systems reveals that the difference between the levels of production is surprisingly more pronounced for the nisin variants (Table 3). The gene replacement approach increases nisin A/Dha5A yields approximately 4 fold and for nisin A/Dha33A the yield is over ten times higher. The increased efficiency of production of the double mutant nisin A/Dha5A,Dha33A is particularly striking (Table 3). When the gene that specifies this variant nisin is plasmid encoded and used to complement the host strains *nisA* deficiency antimicrobial activity was only detected in the more sensitive colony overlay assay. Cell extracts from this strain did not display any activity in plate diffusion bioassays (Table 3). However, when the gene is incorporated into the chromosome using the gene replacement strategy the activity levels were equivalent to that of nisin A representing an increase in production of over 100%. This unexpected finding is of relevance to the subsequent chemical and biochemical analysis of the engineered molecules. Considerable amounts of purified peptides are required to fully characterise the novel nisins and to produce amount on a scale suitable for satisfying the market of a food preservative and the system described here appears to ensure that relatively high yields are achieved.

We have produced a variety of variant nisin-producing strains using the

methodol gy described in this example. These are described in Table 4. Suitable oligonucleotide primers for effecting the specific mutations were designed from the sequence given in Figure 4 and as shown in Figure 11.

Suitable oligonucleotide primers for effecting the specific mutations were designed from the sequence given in Figure 4 and as shown in Figure 11.

Table 4. Nisin producing strains generated by gene replacement.

Strain Number	*nisA* mutation	Activity[a] (% of wt)	MICH[b] ($\mu g\ ml^{-1}$)
MG1614	-	-	-
FI5876	wt	100	0.13
FI7990	Δ*nisA*	-	-
FI8070	S5A	100	0.25
FI8198	S33A	150	0.25
FI8199	S5A,S33A	100	1.00
FI8167	H27W	<1	nd[c]
FI8122	S5A,H27W	10	nd
FI8307	I27K	100	0.13
FI8328	H31K	25	nd
FI8330	H27K,H31K	10	nd
FI8256	K12L	10	0.13
FI8290	ΔM21	<1	nd
FI8289	I30W	<1	0.16

[a] Antimicrobial activity in culture supernatants determined in plate diffusion bioassays.

[b] Minimum inhibitory concentrations (MICs) were determined against the sensitive *L. lactis* strain MG1614, nd, not determined.

In some circumstances it is desirable to add *nisA* nisin as an inducer. Table 5 shows the results of using various inducing agents.

Table 5

Strain	Inducing agent (nisin variant)	Induction[a] 100 ng ml^{-1}	Induction[a] 1 mg ml^{-1}	MIC[b] (mg ml^{-1})
MG1614	-	2	4	-
FI5876	-	94	100	-
FI7847	-	3	3	-
FI7847	A (wild type)	104	104	0.13
FI7847	Dha5A	114	107	0.25
FI7847	Dha33A	17	101	0.25
FI7847	Dha5,33A	34	106	1.00
FI7847	H27K	86	nt	0.13
FI7847	K12L	81	nt	0.13
FI7847	I30W	41	nt	0.16

Example 2: Purification of a variant nisin

Strains FI8070 (*nisA*/S5A) is cultured and the variant nisin (in which Dha5 is replaced with alanine) is secreted into the culture medium.

The variant nisin is purified using a method based on that described by Mulders *et al* (1991) *Eur. J. Biochem.* **201**, 581-584. 1 litre cultures were incubated at 30°C for 16 hours. The pH of cultures was reduced to 2-3 with HCl before centrifugation at 10,000 rpm for 10 minutes. The cell-free supernatants were retained and the pH increased to 5-6 with 10 mM NaOH. To each 10 ml of supernatant 0.99 g of $(NH_4)_2SO_4$ was added.

This solution was then filtered (Millipore, 0.45 μm) prior to running on a Fractogel TSK Butyl 650S (Merk) column, bed volume 5 x 20 cm, previously equilibrated with 0.8 M $(NH_4)_2SO_4$. The column was washed with ~1 litre of 0.8 M $(NH_4)_2SO_4$ until the absorbance at 220 dropped to below 0.5. The bound nisin was eluted with 5 mM HCl and 10 ml fractions were assayed for nisin activity. Active fractions were pooled and freeze dried. Reverse phase HPLC was carried out on the resuspended samples using μBondapak C_{18} column 3.9 x 300 mm run at room temperature. Solvents used were 0.06% (v/v) trifluoroacetic acid and 0.06% (v/v) trifluoroacetic acid in 90% (v/v) aqueous acetonitrile. Absorbance was measured at 220 nm.

Example 3: Addition of variant nisin to cheese

The variant nisin produced by strain FI8070 (in which Dha5 is replaced with alanine) is added at a concentration of 12.5 mg per kg to soft cheese spread in order to prevent the growth of food-spoilage or pathogenic bacteria.

CLAIMS

1. A method for making a cell which does not contain a natural *nisA* gene but expresses a nisin comprising the step of providing a cell with a variant *nisA* gene and genes for nisin modification, secretion and immunity wherein the variant *nisA* gene has the same relationship as the natural *nisA* gene to a gene cluster containing the natural *nisA* gene and the genes for nisin modification, secretion and immunity.

2. A method according to Claim 1 wherein the variant *nisA* gene encodes a variant nisin.

3. A method according to Claim 1 or 2 wherein the variant *nisA* gene comprises a regulatory region other than the natural *nisA* gene regulatory region and a nisin coding region.

4. A method according to any one of Claims 1 to 3 comprising substituting a variant *nisA* gene for the natural, chromosomal *nisA* gene at the chromosomal location of the said natural *nisA* gene.

5. A method according to any one of Claims 1 to 3 comprising providing a gene cluster comprising a variant *nisA* gene and the genes for nisin modification and immunity on an autonomously replicating DNA element.

6. A method according to any one of the preceding claims wherein the cell is a Lactococcus.

7. A method according to Claim 1 or 2 comprising the steps of (1) substituting a counter-selectable *nisA* gene for the natural, chromosomal

nisA gene at the chromosomal location of the said natural *nisA* gene and (2) substituting a variant *nisA* gene for the counter-selectable *nisA* gene at the chromosomal location of the said natural *nisA* gene.

8. A method according to any one of the preceding claims comprising a subsequent step of selecting a cell that is immune to nisin.

9. A method according to Claim 7 or 8 wherein the counter-selectable *nisA* gene comprises an antibiotic resistance gene.

10. A method according to Claim 9 further comprising the step of selecting a cell that is sensitive to the said antibiotic.

11. A method according to any one of the preceding claims wherein the variant *nisA* gene contains a modification to the transcriptional or translational control sequences of the natural *nisA* gene and, as a consequence, the cell expresses an elevated level of its natural *nisA* nisin compared to the natural level.

12. A cell obtainable by the method of any one of the preceding claims.

13. A cell which does not contain a natural *nisA* gene but expresses a nisin comprising a variant *nisA* gene wherein the variant *nisA* gene has the same relationship as the natural *nisA* gene to a gene cluster containing the natural *nisA* gene and the genes for nisin modification, secretion and immunity.

14. A cell according to Claim 13 wherein the variant *nisA* gene encodes a variant nisin.

15. A cell according to Claim 13 r 14 wherein the variant *nisA* gene comprises a regulatory region other than the natural *nisA* gene regulatory region and a nisin coding region.

16. A cell according to any one of Claims 13 to 15 wherein the natural, chromosomal *nisA* gene or part thereof is absent and the cell comprises a variant *nisA* gene at the chromosomal location of the said natural *nisA* gene.

17. A cell according to any one of Claims 13 to 15 comprising an autonomously replicating DNA element carrying a variant *nisA* gene and the genes for nisin modification, secretion and immunity.

18. A cell according to any one of Claims 13 to 17 wherein the cell is a Lactococcus.

19. A cell according to any one of Claims 13 to 18 that is immune to nisin.

20. A cell according to any one of Claims 13 to 19 wherein the variant *nisA* gene contains a modification to the transcriptional or translational control sequences of the natural *nisA* gene and, as a consequence, the cell expresses an elevated level of its natural *nisA* nisin compared to the natural level.

21. A process for producing nisin comprising culturing a cell according to any one of Claims 12 to 20 and obtaining the nisin produced thereby.

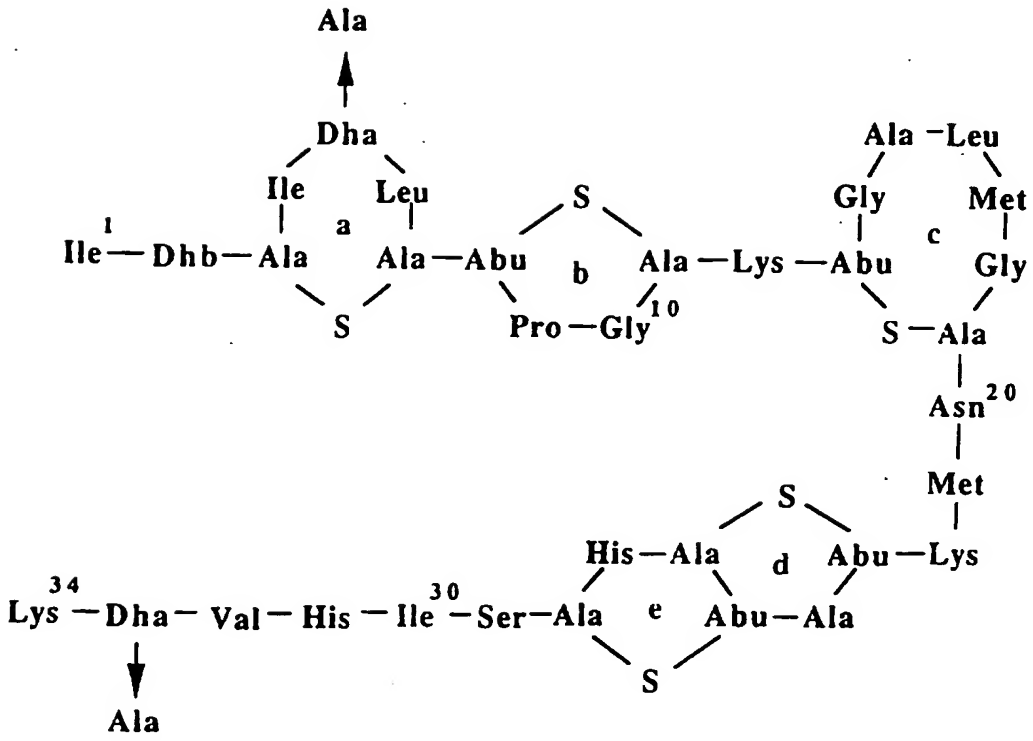
22. A process according to Claim 21 wherein the nisin is a variant nisin.

23. A process according t Claim 21 or 22 wherein the cell is cultured in the presence of *nisA* nisin or a variant nisin which can induce nisin expression.

24. A process according to Claim 23 wherein the amount of *nisA* nisin is a minimum amount that provides maximal induction of nisin production.

25. A nisin produced according to any one of Claims 21 to 24.

26. The use of a nisin according to any one of Claims 21 to 24 as an antimicrobial agent.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Leu
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Gly
Met
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Ala — Abu
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Ala — Lys — Abu
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S
Pro — Gly
S — Ala
Asn
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d
Abu — Lys
Lys — Dha — Val — His — Ile — Ser — Ala
e
Abu — Ala
S
Ala

Figure 1

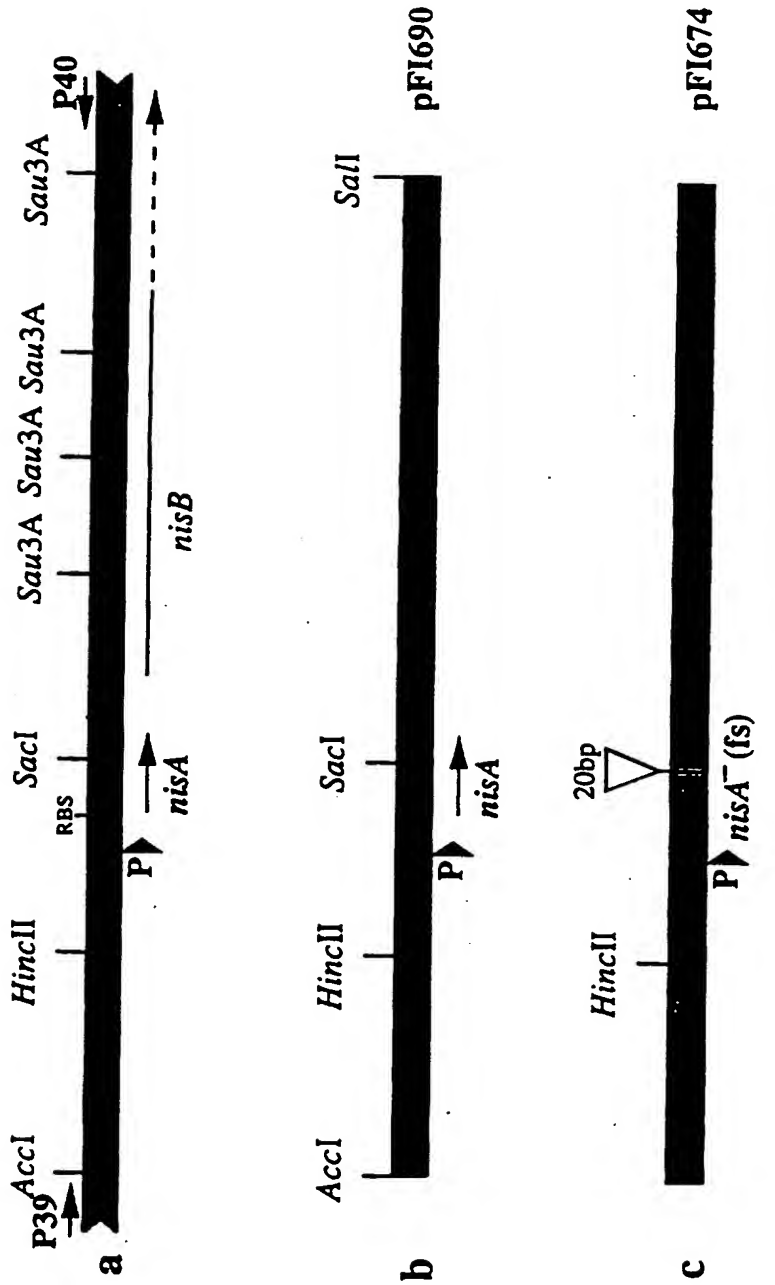


Figure 2 (sheet 1 of 2)

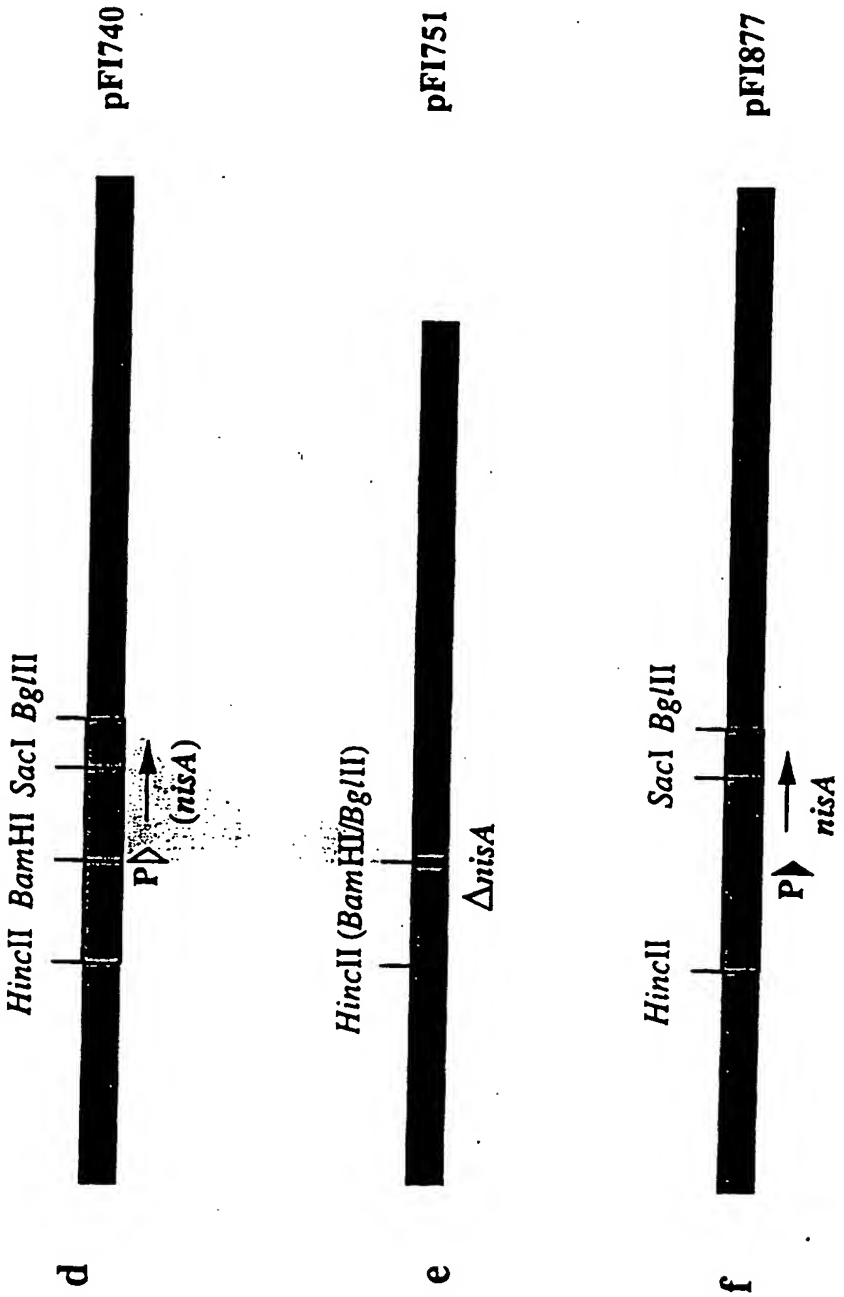
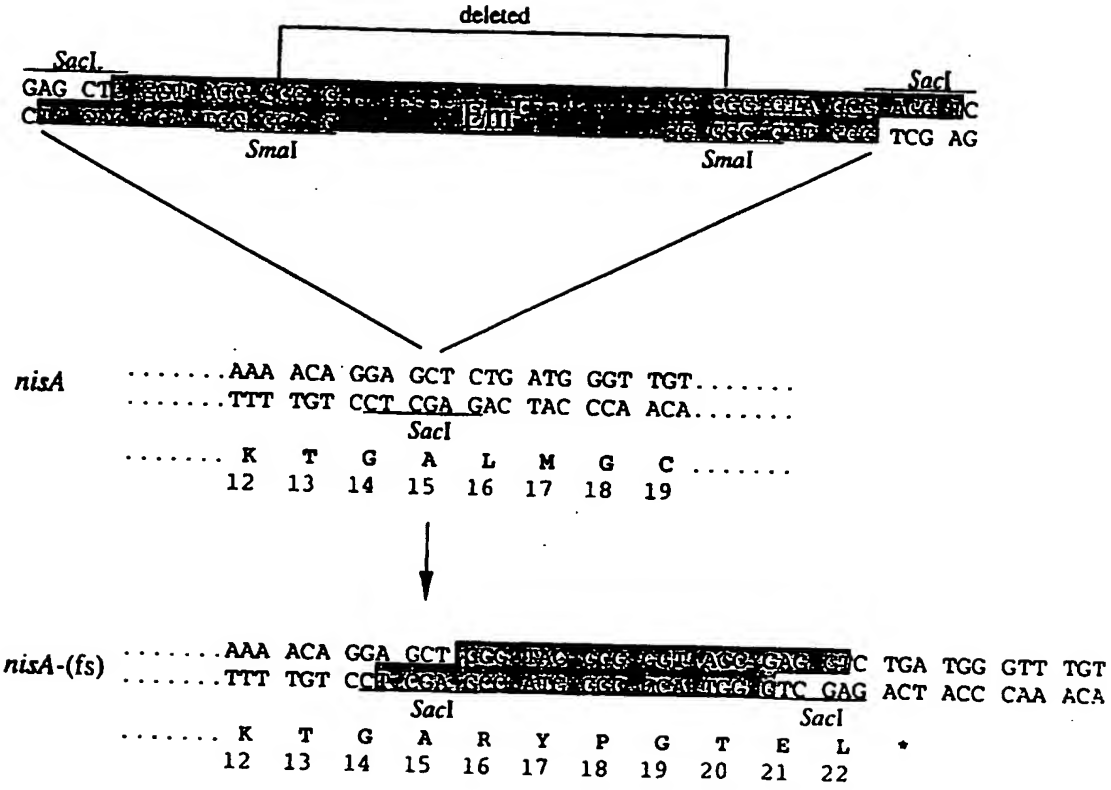


Figure 2 (sheet 2 f 2)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SacI
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TCG AG
SmaI
SmaI
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.....TTT TGT CCT CGA GAC TAC CCA ACA.....
SacI
..... K T G A L M G C .....
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nisA-(fs)
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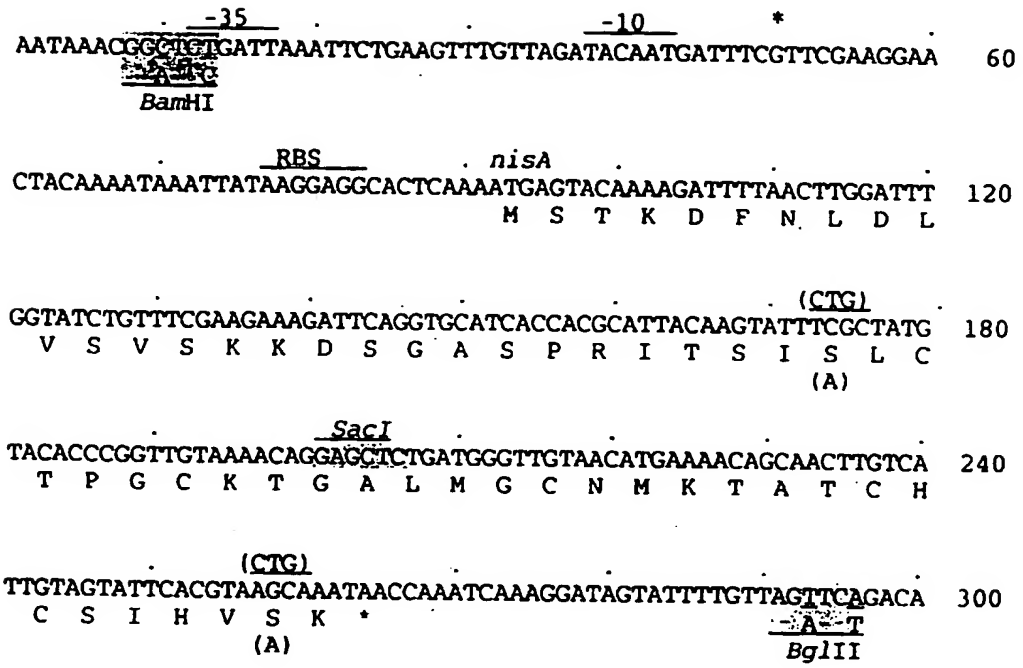
.....TTT TGT CCT CGA
C TGA TGG GTT TGT
GTC GAG ACT ACC CAA ACA
SacI
SacI
..... K T G A R Y P G T E L *
12 13 14 15 16 17 18 19 20 21 22

Figure 3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(CTG)
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(A)
SacI
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(A)
BglII

Figure 4

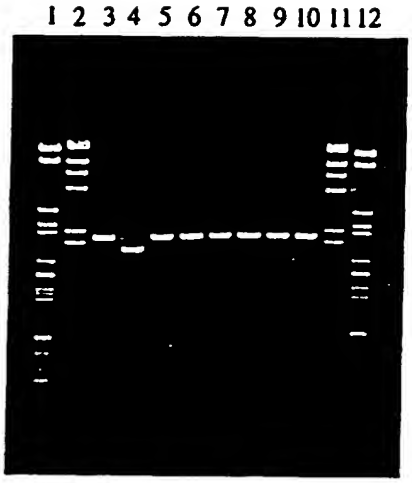
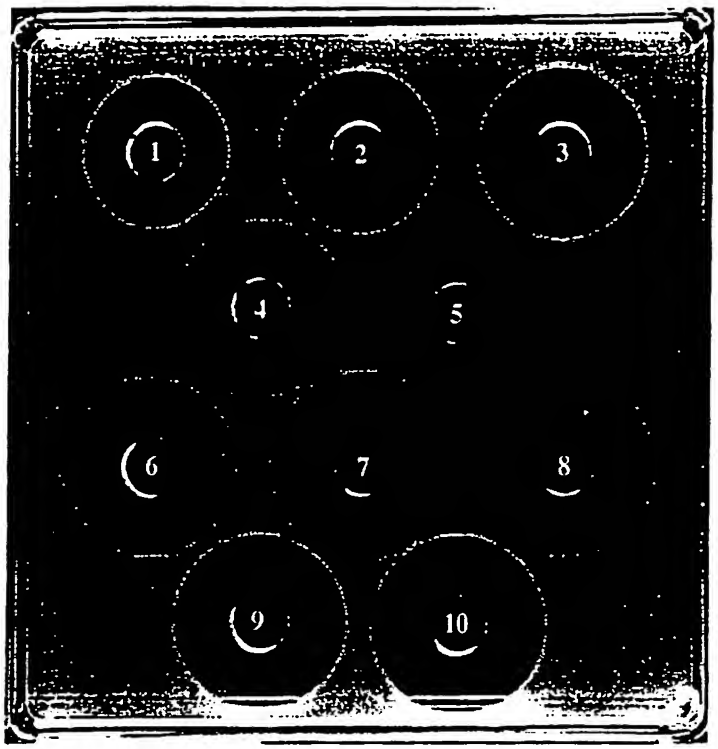


Figure 5



Figur 6

Figure 7 (sheet 1 of 5)

SUBSTITUTE SHEET (RULE 26)

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Figure 7 (sheet 2 f 5)

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        I  Q  N  I  Y  I  I  Y  N  T  S  L  F  M  E  Q  L  F  E  F  L  K  R  E  S  V  V  H  K  K  I  E  D
4500

4501  TACTGAAATATGCAATCAACATATAGGAACTGTTAAAGTAATTAATTTATCATATGTTTACCCTAATTCGAATGCCTTTGCACTAAAGAATATCAATTTA
        T  E  I  C  N  Q  H  I  G  T  V  K  V  I  N  L  S  Y  V  Y  P  N  S  N  A  F  A  L  K  N  I  N  L
4600

4601  TCCTTTGAAAAAGGAGAATTAACTGCTATTGTAGGAAAAAATGGTTCAGGGAAAAGTACACTAGTAAAGATAATTTCAGGATTATATCAACCAACTATGG
        S  F  E  K  G  E  L  T  A  I  V  G  K  N  G  S  G  K  S  T  L  V  K  I  I  S  G  L  Y  Q  P  T  M  G
4700

4701  GAATAATCCAATACGACAAAATGAGAAGTAGTTTGATGCCTGAGGAGTTTTATCAGAAAAACATATCGGTGCTGTTCCAAGATTTTGTGAAGTATGAGTT
        I  I  Q  Y  D  K  M  R  S  S  L  M  P  E  E  F  Y  Q  K  N  I  S  V  L  F  Q  D  F  V  K  Y  E  L
4800
```

Figure 7 (sheet 3 of 5)

```
4801 AACGATAAGAGAGAATATAGGATTGAGTGATTTGTCTTCTCAATGGGAAGATGAGAAAATTATTAAAGTACTAGATAATTTAGGACTCGATTTTTTGAAA 4900
      T  I  R  E  N  I  G  L  S  D  L  S  S  Q  W  E  D  E  K  I  I  K  V  L  D  N  L  G  L  D  F  L  K

4901 ACTAATAATCAATATGTACTTGATACGCAGTTAGGAAATTGGTTTCAAGAAGGGCATCAACTTTCAGGAGGTCAGTGGCAAAAAATTGCATTAGCAAGGA 5000
     T  N  N  Q  Y  V  L  D  T  Q  L  G  N  W  F  Q  E  G  H  Q  L  S  G  G  Q  W  Q  K  I  A  L  A  R  T

5001 CATTCTTTAAGAAAGCTTCAATTTATATTTTAGATGAACCAAGTGCTGCACTCGATCCTGTAGCTGAAAAAGAAATATTTGATTATTTTGTTGCTCTTTC 5100
       F  F  K  K  A  S  I  Y  I  L  D  E  P  S  A  A  L  D  P  V  A  E  K  E  I  F  D  Y  F  V  A  L  S

5101 GGAAAATAATATTTCAATTTTCATTTCTCATAGTTTGAATGCTGCCAGAAAAGCAAATAAAATCGTGGTTATGAAAGATGGACAGGTCGAAGATGTTGGA 5200
      E  N  N  I  S  I  F  I  S  H  S  L  N  A  A  R  K  A  N  K  I  V  V  M  K  D  G  Q  V  E  D  V  G

                                                                      _RBS____
5201 AGTCATGATGTCCTTCTGAGAAGATGTCAATACTATCAAGAACTTTATTATTCAGAGCAATATGAGGATAATGATGAATAAAAAAAATATAAAAAGAAAT 5300
     S  H  D  V  L  L  R  R  C  Q  Y  Y  Q  E  L  Y  Y  S  E  Q  Y  E  D  N  D  E  *
                                                                             nisC -> M  N  K  K  N  I  K  R  N

5301 GTTGAAAAAAATTATTGCTCAATGGGATGAGAGAACTAGAAAAAATAAAGAAAACTTCGATTTCGGAGAGTTGACTCTCTCTACAGGATTGCCTGGTATAA 5400
     V  E  K  I  I  A  Q  W  D  E  R  T  R  K  N  K  E  N  F  D  F  G  E  L  T  L  S  T  G  L  P  G  I  I

5401 TTTTAATGTTAGCGGAGTTAAAAAATAAAGATAACTCAAAGATATATCAGAAAAAGATAGACAATTATATTGAATATATTGTTAGCAAACTTTCAACATA 5500
      L  M  L  A  E  L  K  N  K  D  N  S  K  I  Y  Q  K  K  I  D  N  Y  I  E  Y  I  V  S  K  L  S  T  Y

5501 TGGGCTTTTAACAGGATCACTTTATTCGGGAGCAGCTGGCATTGCATTAAGTATCCTACATTTACGAGAAGATGACGAAAAATATAAGAATCTTCTTGAT 5600
      G  L  L  T  G  S  L  Y  S  G  A  A  G  I  A  L  S  I  L  H  L  R  E  D  D  E  K  Y  K  N  L  L  D

5601 AGCCTAAATAGATATATCGAATATTTCGTCAGAGAAAAAATTGAAGGATTTAATTTGGAAAACATTACTCCTCCTGATTATGACGTGATTGAAGGTTTAT 5700
     S  L  N  R  Y  I  E  Y  F  V  R  E  K  I  E  G  F  N  L  E  N  I  T  P  P  D  Y  D  V  I  E  G  L  S

5701 CTGGGATACTTTCCTATCTATTATTAATCAACGACGAGCAATATGATGATTTGAAAATACTCATTATCAATTTTTTATCAAATCTGACTAAAGAAAACAA 5800
      G  I  L  S  Y  L  L  L  I  N  D  E  Q  Y  D  D  L  K  I  L  I  I  N  F  L  S  N  L  T  K  E  N  N

5801 TGGACTAATATCGCTTTACATCAAATCGGAGAATCAGATGTCTCAATCAGAAAGTGAGATGTATCCACTAGGCTGTTTGAATATGGGATTAGCACATGGA 5900
      G  L  I  S  L  Y  I  K  S  E  N  Q  M  S  Q  S  E  S  E  M  Y  P  L  G  C  L  N  M  G  L  A  H  G

5901 CTTGCTGGAGTGGGCTGTATCTTAGCTTATGCCCACATAAAAGGATATAGTAATGAAGCCTCGTTGTCAGCTTTGCAAAAAATTATTTTTATTTATGAAA 6000
     L  A  G  V  G  C  I  L  A  Y  A  H  I  K  G  Y  S  N  E  A  S  L  S  A  L  Q  K  I  I  F  I  Y  E  K

6001 AGTTTGAACTTGAAAGGAAAAAACAGTTTCTATGGAAAGATGGACTTGTAGCAGATGAATTAAAAAAAGAGAAAGTAATTAGGGAAGCAAGTTTCATTAG 6100
      F  E  L  E  R  K  K  Q  F  L  W  K  D  G  L  V  A  D  E  L  K  K  E  K  V  I  R  E  A  S  F  I  R

6101 AGATGCATGGTGCTATGGAGGTCCAGGTATTAGTCTGCTATACTTATACGGAGGATTAGCACTGGATAATGACTATTTTGTAGATAAAGCAGAAAAAATA 6200
      D  A  W  C  Y  G  G  P  G  I  S  L  L  Y  L  Y  G  G  L  A  L  D  N  D  Y  F  V  D  K  A  E  K  I

6201 TTAGAGTCAGCTATGCAAAGGAAACTTGGTATTGATTCATATATGATTTGCCATGGCTATTCTGGTTTAATAGAAATTTGTTCTTTATTTAAGCGGCTAT 6300
     L  E  S  A  M  Q  R  K  L  G  I  D  S  Y  M  I  C  H  G  Y  S  G  L  I  E  I  C  S  L  F  K  R  L  L
```

Figure 7 (sheet 4 of 5)

```
6301 TAAATACAAAAAAGTTTGATTCATACATGGAAGAATTTAATGTTAATAGTGAGCAAATTCTTGAAGAATACGGAGATGAAAGTGGCACGGGTTTTCTTGA  6400
      N  T  K  K  F  D  S  Y  M  E  E  F  N  V  N  S  E  Q  I  L  E  E  Y  G  D  E  S  G  T  G  F  L  E
                                                                                                  RBS
6401 AGGAATAAGTGGCTGTATACTGGTATTATCGAAATTTGAATATTCAATCAATTTTACTTATTGGAGACAAGCACTGTTACTTTTTGACGATTTTTTGAAA  6500
      G  I  S  G  C  I  L  V  L  S  K  F  E  Y  S  I  N  F  T  Y  W  R  Q  A  L  L  L  F  D  D  F  L  K
6501 GGAGGGAAGAGGAAATGAGAAGATATTTAATACTTATTGTGGCCTTAATAGGGATAACAGGTTTATCAGGGTGTTATCAAACAAGTCATAAAAAGGTGAG  6600
     G  G  K  R  K  *
           nisI -> M  R  R  Y  L  I  L  I  V  A  L  I  G  I  T  G  L  S  G  C  Y  Q  T  S  H  K  K  V  R
6601 GTTTGACGAAGGAAGTTATACTAATTTTATTTATGATAATAAATCGTATTTCGTAACTGATAAGGAGATTCCTCAGGAGAACGTTAACAATTCCAAAGTA  6700
      F  D  E  G  S  Y  T  N  F  I  Y  D  N  K  S  Y  F  V  T  D  K  E  I  P  Q  E  N  V  N  N  S  K  V
6701 AAATTTTATAAGCTGTTGATTGTTGACATGAAAAGTGAGAAACTTTTATCAAGTAGCAACAAAAATAGTGTGACTTTGGTCTTAAATAATATTTATGAGG  6800
     K  F  Y  K  L  L  I  V  D  M  K  S  E  K  L  L  S  S  S  N  K  N  S  V  T  L  V  L  N  N  I  Y  E  A
6801 CTTCTGACAAGTCGCTATGTATGGGTATTAACGACAGATACTATAAGATACTTCCAGAAAGTGATAAGGGGGCGGTCAAAGCTTTGAGATTACAAAACTT  6900
       S  D  K  S  L  C  M  G  I  N  D  R  Y  Y  K  I  L  P  E  S  D  K  G  A  V  K  A  L  R  L  Q  N  F
6901 TGATGTGACAAGCGATATTTCTGATGATAATTTTGTTATTGATAAAAATGATTCACGAAAAATTGACTATATGGGAAATATTTACAGTATATCGGACACC  7000
      D  V  T  S  D  I  S  D  D  N  F  V  I  D  K  N  D  S  R  K  I  D  Y  M  G  N  I  Y  S  I  S  D  T
7001 ACCGTATCTGATGAAGAATTGGGAGAATATCAGGATGTTTTAGCTGAAGTACGTGTGTTTGATTCAGTTAGTGGCAAAAGTATCCCGAGGTCTGAATGGG  7100
     T  V  S  D  E  E  L  G  E  Y  Q  D  V  L  A  E  V  R  V  F  D  S  V  S  G  K  S  I  P  R  S  E  W  G
7101 GGAGAATTGATAAGGATGGTTCAAATTCCAAACAGAGTAGGACGGAATGGGATTATGGCGAAATCCATTCTATTAGAGGAAAATCTCTTACTGAAGCATT  7200
       R  I  D  K  D  G  S  N  S  K  Q  S  R  T  E  W  D  Y  G  E  I  H  S  I  R  G  K  S  L  T  E  A  F
                                              RBS
7201 TGCCGTTGAGATAAATGATGATTTTAAGCTTGCAACGAAGGTAGGAAACTAGAGTGAAAAAAATACTAGGTTTCCTTTTTATCGTTTGTTCGTTGGGTTT  7300
      A  V  E  I  N  D  D  F  K  L  A  T  K  V  G  N  *
                                                      nisP -> V  K  K  I  L  G  F  L  F  I  V  C  S  L  G  L
7301 ATCAGCAACTGTGCATGGGGAGACAACAAATTCACAACAGTTACTCTCAAATAATATTAATACGGAATTAATTAATCATAATTCTAATGCAATTTTATCT  7400
      S  A  T  V  H  G  E  T  T  N  S  Q  Q  L  L  S  N  N  I  N  T  E  L  I  N  H  N  S  N  A  I  L  S
7401 TCAACAGAGGGATCAACGACTGATTCGATTAATCTAGGGGCGCAGTCACCTGCA  -->                                           7554
     S  T  E  G  S  T  T  D  S  I  N  L  G  A  Q  S  P  A   etc
```

Figure 7 (sheet 5 of 5)

Figure 8 (sheet 1 of 4)

```
1401  AAAATCAAAAAGTAGAGGCACAACCTCTATTGATAAGTAATTCTTCTGAAAAGAAAGCAAGTGTTTATACAAATTCACATGATTTTTGGGATTATCAGTG   1500
        N  Q  K  V  E  E  A  Q  P  L  L  I  S  N  S  S  E  K  K  A  S  V  Y  T  N  S  H  D  F  W  D  Y  Q  W

1501  GGATATGAAATATGTGACAAATAATGGAGAAAGCTATGCGCTCTACCAGCCCTCAAAGAAAATTTCTGTTGGAATTATTGATTCAGGAATCATGGAAGAA   1600
        D  M  K  Y  V  T  N  N  G  E  S  Y  A  L  Y  Q  P  S  K  K  I  S  V  G  I  I  D  S  G  I  M  E  E

1601  CATCCTGATTTGTCAAATAGTTTAGGAAATTATTTTAAAAATCTTGTTCCTAAGGGAGGGTTTGATAATGAAGAACCTGATGAAACTGGAAATCCAAGTG   1700
        H  P  D  L  S  N  S  L  G  N  Y  F  K  N  L  V  P  K  G  G  F  D  N  E  E  P  D  E  T  G  N  P  S  D

1701  ATATTGTCGACAAAATGGACACGGACGGAAGTCGCAGGTCAGATTACAGCCAAATGGTAATATTTTAGGAGTAGCACCAGGGATTACTGTAAATATATA   1800
        I  V  D  K  M  G  H  G  T  E  V  A  G  Q  I  T  A  N  G  N  I  L  G  V  A  P  G  I  T  V  N  I  Y

1801  CAGAGTATTTGGTGAAAATCTTTCGAAATCGGAATGGGTAGCTAGAGCAATAAGAAGAGCTGCGGATGATGGGAACAAGGTCATCAATATAAGTGCTGGA   1900
        R  V  F  G  E  N  L  S  K  S  E  W  V  A  R  A  I  R  R  A  A  D  D  G  N  K  V  I  N  I  S  A  G

1901  CAGTATCTTATGATTTCAGGATCGTATGATGGAACAAATGATTATCAAGAGTATCTTAATTATATAAGTCAGCAATAAATTATGCAACAGCAAAAGCGAA   2000
        Q  Y  L  M  I  S  G  S  Y  D  D  G  T  N  D  Y  Q  E  Y  L  N  Y  K  S  A  I  N  Y  A  T  A  K  G  S

2001  GTATTGTTGTCGCAGCTCTTGGTAATGATAGTTTAAACATACAAGATAACCAAACACAATGATAAACTTTCTTAAGCGTTTCAGAAGTATAAAGGTTCCTGG   2100
        I  V  V  A  A  L  G  N  D  S  L  N  I  Q  D  N  Q  T  M  I  N  F  L  K  R  F  R  S  I  K  V  P  G

2101  AAAAGTTGTAGATGCACCGAGTGTATTTGAGGATGTAATAGCCGTAGGTGGAATAGATGGTTATGGTAATATTTCTGATTTTAGTAATATTGGAGCGGAT   2200
        K  V  V  D  A  P  S  V  F  E  D  V  I  A  V  G  G  I  D  G  Y  G  N  I  S  D  F  S  N  I  G  A  D

2201  GCAATTTATGCTCCTGCTGGCACAACGGCCAATTTTAAAAATATGGGCAAGATAAATTTGTCAGTCAGGGTTATTATTTGAAAGATTGGCTTTTTACAA   2300
        A  I  Y  A  P  A  G  T  T  A  N  F  K  K  Y  G  Q  D  K  F  V  S  Q  G  Y  Y  L  K  D  W  L  F  T  T

2301  CTGCTAATACTGGCTGGTACCAATATGTTTATGGCAACTCATTTGCTACTCCTAAAGTATCTGGGCACTGGCATTAGTAGTTGATAAATATGGAATAAA   2400
        A  N  T  G  W  Y  Q  Y  V  Y  G  N  S  F  A  T  P  K  V  S  G  A  L  A  L  V  V  D  K  Y  G  I  K
                                          EcoRI
2401  GAATCCTAACCAACTAAAAAGGTTTCTTCTAATGAATTCTCCAGAAGTTAATGGGAATAGAGTATTGGAATATTGTTGATTTATTGAATGGGAAAAATAAA   2500
        N  P  N  Q  L  K  R  F  L  L  M  N  S  P  E  V  N  G  N  R  V  L  N  I  V  D  L  L  N  G  K  N  K
  HindIII
2501  GCTTTTAGCTTAGATACAGATAAAGGTCAGGATGATGCTATTAACCATAAATCGATGGAGAATCTTAAAGAGTCTAGGGATACAATGAAACAGGAACAAG   2600
        A  F  S  L  D  T  D  K  G  Q  D  D  A  I  N  H  K  S  M  E  N  L  K  E  S  R  D  T  M  K  Q  E  Q  D

2601  ATAAAGAAATTCAAAGAAATACAAATAACAATTTTCTATCAAAAATGATTTTCATAACATTTCAAAAGAAGTAATTTCAGTTGATTATAATATTAATCA   2700
        K  E  I  Q  R  N  T  N  N  N  F  S  I  K  N  D  F  H  N  I  S  K  E  V  I  S  V  D  Y  N  I  N  Q

2701  AAAAATGGCTAATAATCGAAATTCGAGAGGTGCTGTTTCTGTACGAAGTCAAGAAATTTTACCTGTTACTGGAGATGGAGAAGATTTTTTACCGGCTTTA   2800
        K  M  A  N  N  R  N  S  R  G  A  V  S  V  R  S  Q  E  I  L  P  V  T  G  D  G  E  D  F  L  P  A  L

2801  GGTATAGTGTGTATCTCAATCCTTGGTATATTGAAAAGAAAGACTAAAAAATTGATAGATTATATTTCTTCAGAATGAATGGTATAATGAAGTAATGAGTA   2900
        G  I  V  C  I  S  I  L  G  I  L  K  R  K  T  K  N  *
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Figure 8 (sheet 2 of 4)

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                    RBS
2901  CTAAACAATCGGAGGTAAAGTGGTGTATAAAATTTTAATAGTTGATGATCAGGAAATTTTAAAATTAATGAAGACAGCATTAGAAATGAGAAACTAT
                nisR -> V V Y K I L I V D D D Q E I L K L M K T A L E M R N Y                              3000
                                                    EcoRV
3001  GAAGTTGCGACGCATCAAAACATTTCACTTCCCTTGGATATTACTGATTTCAGGGATTTGATTTGTTAGATATCATGATGTCAAATATTGAAG
        E V A T H Q N I S L P L D I T D F Q G F D L I L L L D I M M S N I E G                                3100

3101  GGACAGAAATTTGTAAAAGGATTCGCAGAGAAATATCAACTCCAATTATCTTTGTTAGTGCGAAAGATACAGAAGAGGATATTATAAACGGCTTAGGTAT
          T E I C K R I R R E I S T P I I F V S A K D T E E D I I N G L G I                                  3200

3201  TGGTGGGGATGACTATATTACTAAGCCTTTTAGCCTTTAAACAGTTGGTTGCAAAAGTGGAAGCAAATATAAAGCGAGAGGAACGCAATAAACATGCAGTT
        G G D D Y I T K P F S L K Q L V A K V E A A N I K R E E R N K H A V                                  3300

3301  CATGTTTTTTCAGAGATTCGTAGAGATTTAGGACCAATTACATTTTATTTAGAAGAAAGGCGAGTCTGTGTCAATGGTCAAACAATTCCACTGACTTGTC
        H V F S E I R R D L G P I T F Y L E E R R V C V N G Q T I P L T C R                                  3400

3401  GTGAATACGATATTCTTGAATTACTATCACAACGAACTTCTAAAGTTTATACGAGAGAGGATATTTATGATGACGTATATGATGAATATTCTAATGCACT
          E Y D I L E L L S Q R T S K V Y T R E D I Y D D V Y D E Y S N A L                                  3500
                                                                                    RBS
3501  TTTTCGGTCAATCTCGGAGTATATTTATCAGATTAGGAGTAAGTTTGCACCATACGATATTAATCCGATAAAAACGGTTCGGGGACTTGGGTATCAGTGG
        F R S I S E Y I Y Q I R S K F A P Y D I N P I K T V R G L G Y Q W                                    3600

3601  CATGGGTAAAAAAATATTCAATGCGTCGACGGATATGGCAAGCTGTCATTGAAATTATCATAGGTACTTGTCTACTTATCCTGTTGTTACTGGGCTTGACT
        H G *
nisK -> M G K K Y S M R R R I W Q A V I E I I I G T C L L I L L L G L T                                    3700

3701  TTCTTTCTACGACAAATTGGACAAATCAGTGGTTCAGAAACTATTCGTTTATCTTTAGATTCAGATAATTTAACTATTTCTGATATCGAACGTGATATGA
        F F L R Q I G Q I S G S E T I R L S L D S D N L T I S D I E R D M K                                  3800

3801  AACACTACCCATATGATTATATTATTTTTGACAATGATACAAGTAAAATTTTGGGAGGACATTATGTCAAGTCGGATGTACCTAGTTTTGTAGCTTCAAA
          H Y P Y D Y I I F D N D T S K I L G G H Y V K S D V P S F V A S K                                  3900

3901  ACAGTCTTCACATAATATTACAGAAGGAGAAATTACTTATACTTATTCAAGCAATAAGCATTTTTCAGTTGTTTTAAGACAAAACAGTATGCCTGAATTT
        Q S S H N I T E G E I T Y T Y S S N K H F S V V L R Q N S M P E F                                    4000
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Figure 8 (she t 3 or 4)

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4001  ACAAATCATACGCTTCGTTCAATTTCTTATAATCAATTTACTTACCTTTTCTTTTTTCTTGGTGAAATAATACTCATTATTTTTTCTGTCTATCATCTCA
        T  N  H  T  L  R  S  I  S  Y  N  Q  F  T  Y  L  F  F  F  L  G  E  I  I  L  I  I  F  S  V  Y  H  L  I
4101  TTAGAGAATTTTTCTAAGAATTTTCAAGCCGTTCAAAAGATTGCATTGAAGATGGGGGAAATAACTACTTTTCCTGAACAAGAGGAATCAAAAATTATTGA
        R  E  F  S  K  N  F  Q  A  V  Q  K  I  A  L  K  M  G  E  I  T  T  F  P  E  Q  E  E  S  K  I  I  E
4201  ATTTGATCAGGTTCTGAATAACTTATATATTCGAAAAGTAAGGAGTTAGCTTTCCTTATTGAAGCGGAGCGTCATGAAAAACATGATTTATCCTTCCAGGTT
        F  D  Q  V  L  N  N  L  Y  S  K  S  K  E  L  A  F  L  I  E  A  E  R  H  E  E  K  H  D  L  S  F  Q  V
4301  GCTGCACTTTCACATGATGTTAAGACACCTTTAACAGTATTAAAAGGAAATATTGAACTGCTAGAGATGACTGAAGTAAATGAACAACAAGCTGATTTTA
        A  A  L  S  H  D  V  K  T  P  L  T  V  L  K  G  N  I  E  L  L  E  M  T  E  V  N  E  Q  Q  A  D  F  I
4401  TTGAGTCAATGAAAAATAGTTTGACTGTTTTTGACAAGTATTTTAACACAATGATTAGTTATACAAAACTTTTGAATGATGAAAATGATTACAAAGCGAC
        E  S  M  K  N  S  L  T  V  F  D  K  Y  F  N  T  M  I  S  Y  T  K  L  L  N  D  E  N  D  Y  K  A  T
4501  AATCTCCCTGGAGGATTTTTTGATAGATTTATCAGTTGAGTTGGAAGAGTTGTCAACAACTTATCAAGTGGATTATCAGCTAGTTAAAAAAACAGATTTA
        I  S  L  E  D  F  L  I  D  D  L  S  V  E  L  E  E  L  S  T  T  Y  Q  V  D  Y  Q  L  V  K  K  T  D  L
4601  ACCACTTTTTACGGAAATACATTAGCTTTAAGTCGAGCACTTATCAATATCTTTGTTAATGCCTGTCAGTATGCTAAAGAGGGTGAAAAATAGTCAGTT
        T  T  F  Y  G  N  T  L  A  L  S  R  A  L  I  N  I  F  V  N  A  C  Q  Y  A  K  E  G  E  K  I  V  S  L
4701  TGAGTATTTATGATGATGAAAAATATCTCTATTTTGAAATCTGGAATAATGGTCATCCTTTTTCTGAACAAGCAAAAAAAAATGCTGGAAAACTATTTTT
        S  I  Y  D  D  E  K  Y  L  Y  F  E  I  W  N  N  G  H  P  F  S  E  Q  A  K  K  N  A  G  K  L  F  F
4801  CACAGAAGATACTGGACGTAGTGGGAAACACTATGGGATTGGACTATCTTTTGCTCAAGGTGTAGCTTTAAAACATCAAGGAAACTTAATTCTCAGTAAT
        T  E  D  T  G  R  S  G  K  H  Y  G  I  G  L  S  F  A  Q  G  V  A  L  K  H  Q  G  N  L  I  L  S  N
4901  CCTCAAAAAGGTGGGGCAGAAGTTATCCTAAAAATAAAAAAGTAATTTAGTAATCTCTAAGGATTACTTTTTTTGTTTCTGAATAGATTCTGAAAATTGT
        P  Q  K  G  G  A  E  V  I  L  K  I  K  K  *
```

Figure 8 (sheet 4 of 4)

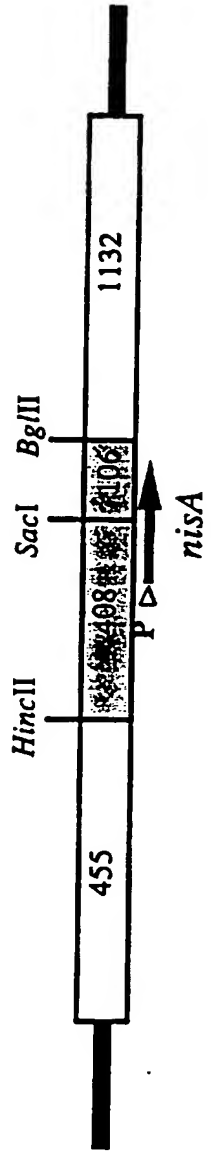

Figure 9

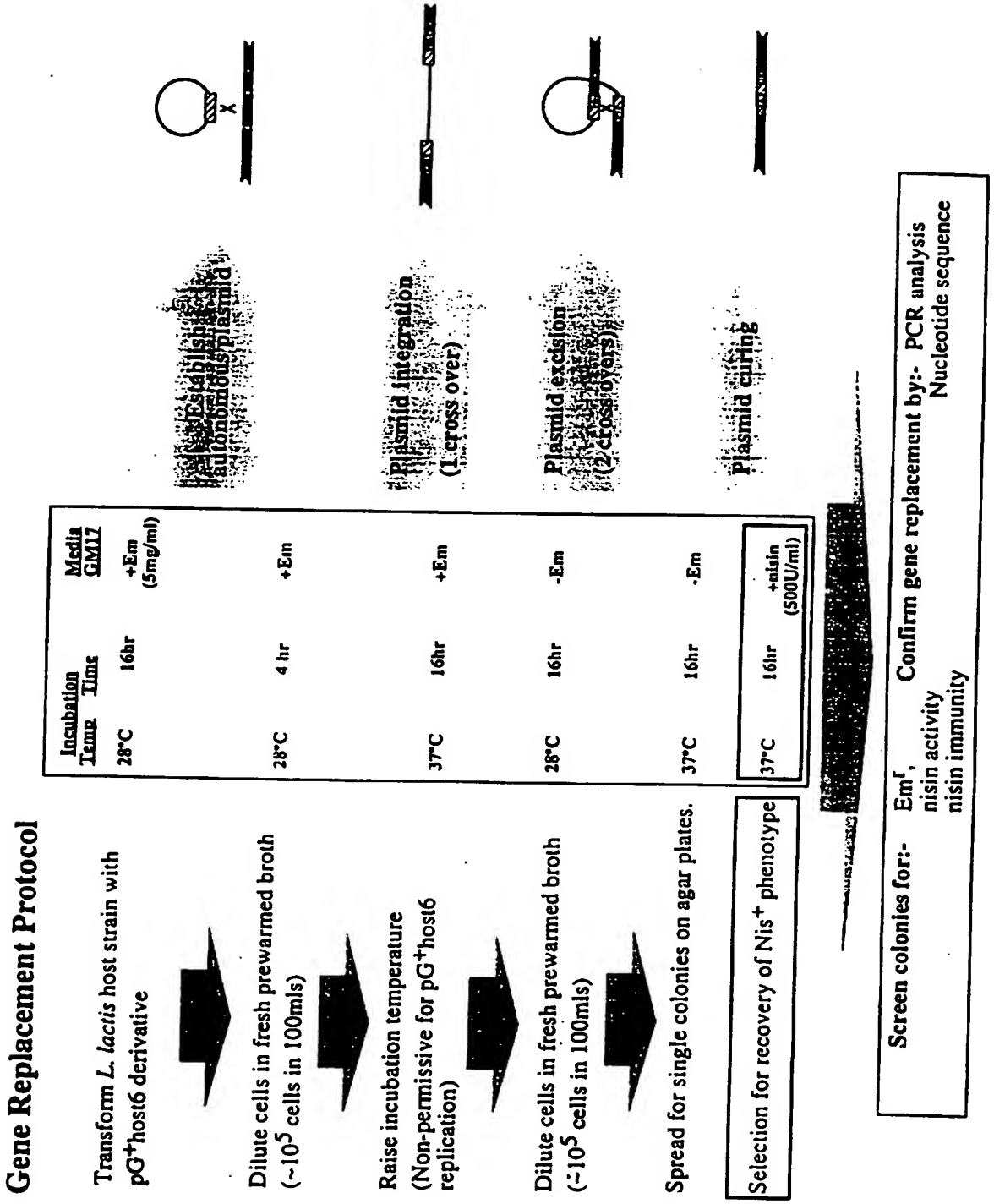
Gene Replacement Protocol
Transform L. lactis host strain with pG+host6 derivative
Dilute cells in fresh prewarmed broth (~10^5 cells in 100mls)
Raise incubation temperature (Non-permissive for pG+host6 replication)
Dilute cells in fresh prewarmed broth (~10^5 cells in 100mls)
Spread for single colonies on agar plates.
Selection for recovery of Nis+ phenotype
Incubation Temp Time Media GM17
28°C 16hr +Em (5mg/ml)
28°C 4 hr +Em
37°C 16hr +Em
28°C 16hr -Em
37°C 16hr -Em
37°C 16hr +nisin (500U/ml)
Establishment of autonomous plasmid
Plasmid integration (1 cross over)
Plasmid excision (2 cross overs)
Plasmid curing
Screen colonies for:- Emr, nisin activity nisin immunity
Confirm gene replacement by:- PCR analysis Nucleotide sequence

Figure 10

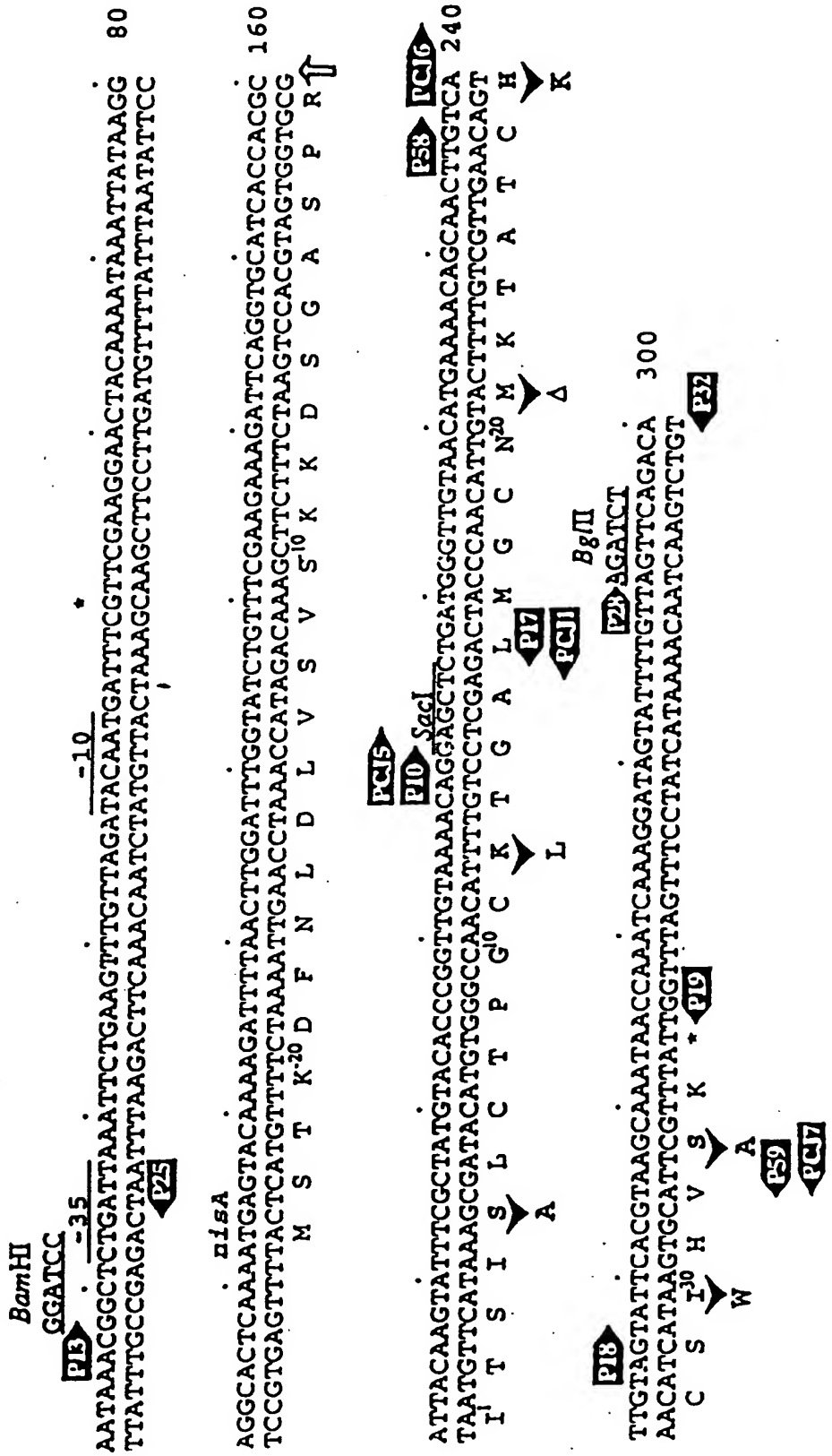

Figure 11

SUBSTITUTE SHEET (RULE 26)

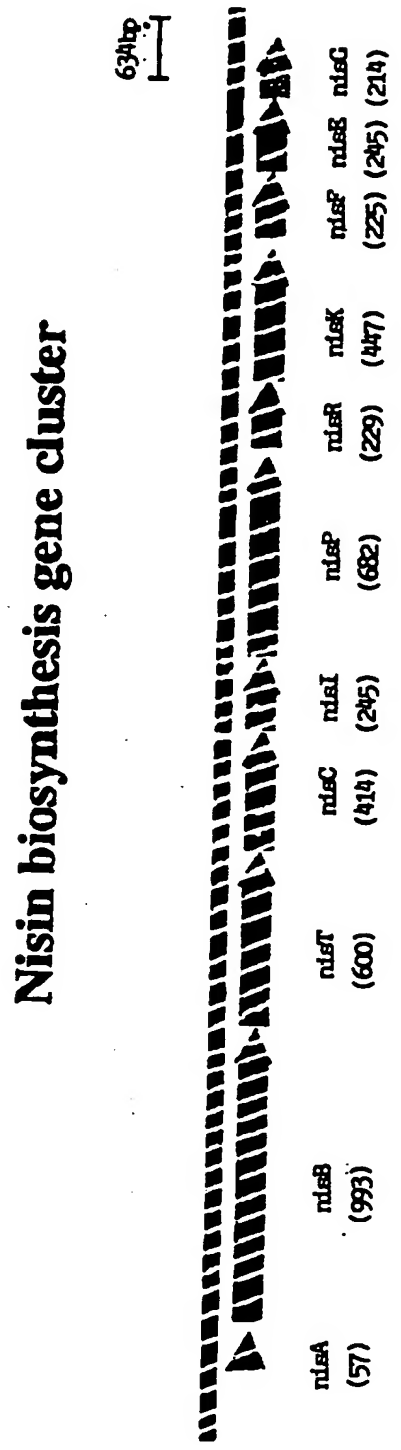
Nisin biosynthesis gene cluster
634bp
nisA (57)
nisB (993)
nisT (600)
nisC (414)
nisI (245)
nisP (682)
nisR (229)
nisK (447)
nisF (225)
nisE (245)
nisG (214)

Figure 12

INTERNATIONAL SEARCH REPORT

In: nal Application No
PCT/GB 95/02699

A. CLASSIFICATION F SUBJECT MATTER
IPC 6 C12N15/74 C07K14/315 C12P21/02 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 18633 (STICHTING NEDERLANDS INSTITUUT VOOR ZUIVELONDERZOEK (NIZO)) 29 October 1992 cited in the application *Examples 7-9* ---	1-3,5,6, 11-15, 17-22,25
X	EP,A,0 137 869 (MICROLIFE TECHNICS, INC.) 24 April 1985 *Examples 1-7* --- -/--	1-3,6, 12-15, 18,19, 21,22, 25,26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 March 1996	28.03.96

Name and mailing address of the ISA	Authorized officer
European Patent ffice, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Alt, G

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATO ASI.SERL, SER.H, vol. 65, 1992 pages 449-461, DE VUYST, L. AND VANDAMME, E.J. 'Localization and phenotypic expression of genes involved in the biosynthesis of the Lactococcus lactis subsp.lactis lantibiotic nisin' *pages 454-455:"Localization of the nisA gene"*	1
A	JOURNAL OF BACTERIOLOGY, vol. 174, no. 4, February 1992 pages 1280-1287, RAUCH, P.J.G. AND DE VOS, W.M. 'Characterization of the novel nisin-sucrose conjugative transposonTn5376 and its insertion in Lactococcus lactis' *Abstract*	1,13
A	WO,A,93 20213 (AGRICULTURE AND FOOD RESEARCH COUNCIL; GASSON, M.J. AND DODD, H.M.) 14 October 1993 cited in the application *pages 19-29*	1,13
A	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 58, no. 11, 1 November 1992 pages 3683-3693, DODD, H.M. ET AL. 'A lactococcal expression system for engineered nisins' cited in the application *whole document*	1,13
A	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 55, no. 2, 1 February 1989 pages 394-400, LEENHOUTS, K. ET AL. 'Campbell-like integration of heterologous plasmid DNA into the chromosome of Lactococcus lactis subsp. lactis' cited in the application *pages 397-398:"Analysis of transformants obtained with pHV60 carrying chromsomal inserts"*	1

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BACTERIOLOGY, vol. 175, no. 11, 1 June 1993 pages 3628-3635, BISWAS, I. ET AL. 'High-efficiency gene inactivation and replacement system for gram-positive bacteria' cited in the application *pages 3628-3629:"Construction of plasmids for integration"; page 3632, left-hand column, second paragraph to right-hand column, first paragraph* ---	1
P,X	MICROBIOLOGY, vol. 142, no. 1, 1 January 1996 pages 47-55, DODD, H.M. ET AL. 'A gene replacement strategy for engineering nisin' *whole document* -----	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir. ...nal Application No
PCT/GB 95/02699

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		CA-C-	1220738	21-04-87
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		JP-A-	60066976	17-04-85
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		US-A-	4740593	26-04-88
		US-A-	4716115	29-12-87
WO-A-9320213	14-10-93	CA-A-	2133578	14-10-94
		EP-A-	0633939	18-01-95
		GB-A,B	2280442	01-02-95